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Inhibitors of TGF-beta/TGF-R signaling for treatment of neurodegenerative diseases

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Inhibitors of TGF-beta/TGF-R signaling for treatment of neurodegenerative diseases

The present invention relates to the use of a compound interfering with (a) the biological activity of TGF-beta1 or its expression or (b) the TGF-beta1/TGF-R signaling, for the preparation of a pharmaceutical composition for the prevention or treatment of a disease, wherein neurogenesis and/or neuroregeneration has a beneficial effect, in particular a disease like Morbus Alzheimer, Morbus Parkinson, Lewy Body Dementia, Amytrophic Lateral Sclerosis, Spinocerebellar Atrophies, Creutzfeldt Jakobs Disease, Frontemporal Dementia, Morbus Pick, AIDS Dementia Complex, Vascular Dementia, Progressive Supranuclear Palsy, Corticobasal Degeneration, Multisystem-Atrophy, Hallervorden Spatz Disease, Huntington`s disease, Stroke, Traumatic Brain Injury, Retinitis Pigmentosa, Macular Degeneration, Glaucoma, Depression, Schizophrenia, and Multiple Sclerosis.

A number of severe neurodegenerative disorders have severe socioeconomic impact upon modern societies, e.g., disorders like Morbus Alzheimer, Morbus Parkinson, Lewy Body Dementia, Frontemporal Dementia, Morbus Pick, Amytrophic Lateral Sclerosis, Spinocerebellar Atrophies, Creutzfeldt Jakobs Disease, AIDS Dementia Complex, Vascular Dementia, Progressive Supranuclear Palsy, Corticobasal Degeneration, Multisystem-Atrophy, Hallervorden Spatz Disease, Huntington`s disease, Stroke, Traumatic Brain Injury, Retinitis Pigmentosa, Macular Degeneration, Glaucoma, Depression, Schizophrenia, and Multiple Sclerosis. The common pathophysiological cause is found in genetic or epigenetic defects ultimately leading to progressive dysfunction and finally to neuronal or glial cell death and disintegration. Microglia cells and perivascular resting macrophages are attracted and activated trying to clear the cell and tissue debris. This may happen in a very

short span of time, as in Creutzfeldt Jacobs Disease, or over decades, as in Parkinson's Disease. The activated microglial / macrophage cell population releases a number of inflammatory cytokines into the extracellular matrix, draining either into small venules or the CSF-space.

Unfortunately, neurogenesis and neuroregeneration that could have an advantageous effect on the diseases described above is suppressed by so far unknown mechanisms.

Thus, the technical problem underlying the present invention is to provide means suitable for treating or preventing neurodegenerative disorders or at least symptoms associated with said disorders by interfering with the suppression of neurogenesis and neuroregeneration.

The solution of the said technical problem is achieved by providing the embodiments characterized in the claims. The TGF-beta family of proteins, namely TGF-beta1, TGF-beta2 and TGF-beta3 with their specific cell surface receptors TGF-$R_{I,II,III}$ are known to act on several crucial aspects of embryonal and mainly mesenchymal/neuroektodermal organ development (for review see Johnson, Jennings et al. 1993; Bottner, Krieglstein et al., 2000)). They allow embryonal stem cells to differentiate into neuronal precursor cells, and are neuroprotective for injured mature neurons (for review see Unsicker and Krieglstein, 2002). It is further known that they have a critical impact upon hematopoetic stem cell differentiation, controlling proliferation and also differentiation (for review see Ruscetti and Bartelmez 2001). During the experiments leading to the present invention it was found that TGF-beta1 is a crucial factor involved in suppression of neurogenesis and neuroregeneration and, accordingly, a compound which is capable of interfering with this biological activity of TGF-beta1 is useful for the

treatment/prevention of the diseases discussed above. TGF-beta1, by interacting with the TGF-betaR present on CNS precursor / stem cells, is a crucial modulator of cellular CNS repair. Described is a regulatory mechanism, by which neural stem cell activity is modulated by the control of TGF-beta present in the surrounding extracellular environment. Under normal, physiological conditions, low levels of TGF-beta1 allow continuous neurogenesis in neurogenic regions of the adult brain. Under pathological conditions, however, TGF-beta levels are increased, mainly due to microglial/macrophage activation and due to activation of latent TGF-beta, causing a down-regulation of neural stem cell activity and the impairment of cellular replacement and neuroregeneration. The present invention has profound socioeconomic consequences: most disorders mentioned in the context above lead to relatively longstanding disability, put people out of job or render them care-dependant, all being very cost-intensive for society, insurance companies, and health-care suppliers. Modulating these disorders in favour of neuro-repair and neuroregeneration will mean normal or almost normal quality of life for the patients, depending on the efficacy of treatment. It will also mean a very significant cost reduction for society, and most importantly, return of those individuals affected to a productive life style.

To summarize, as a result of the experiments leading to the present invention (1) a physiological regulatory circuit controlling neurogenesis and neuro-repair was found, (2) it could be shown that the regulation takes place via fluid compartments, which have direct contact to neuronal cells and their precursors and (3) an inhibitory circuit has been shown as being an ideal target for strategies to repair damage within the CNS, overwhelmingly being applicable for almost all destructive pathology in the nervous system. So far it has been tried to increase rather than decrease (see v.) TGF-ß

function in order to augment its known neuroprotective activities. (4) Although for a long time it has been speculated that inflammatory processes play a significant role in neurodegeneration, and a relative large amount of preclinical and clinical data seem to support this idea, the master circuit is now being described, that orchestrates all the single regulatory sub-circles, e.g. cytokines (IL-1, IL-6,IL-12) and others. (5) In addition, it might be noted that Nature has installed neuroprotection above neuroregeneration: it has not been shown so far that the immunopriviliged and highly protected CNS (protected especially against immune attacks), which is in significant part due to the TGF-ß system, has deficits in neuroregeneration due to this privilege and due to the same molecule TGF-ß. The evolutionary concept seems to argue in favour for a highly sophisticated CNS and its most complex functioning; in this context, individual neuroregeneration seems less important for evolution.

Brief description of the drawings

Figure 1: TGF-beta1 inhibits proliferation of adult rodent neural stem and precursor cells

Adult rodent neural stem and precursor cell (NSC) cultures were treated with various concentrations (0, 5, 10, 50 ng/ml) of recombinant human TGF-beta1 for 7 days. On day 7 viable cells were counted by trypan blue exclusion assay in a hemocytometer. The data are expressed as average ± SD from three experiments performed in triplicate.

Figure 2: TGF-beta2 does not interfere with proliferation of adult rodent NSCs

Adult rodent NSC cultures were treated with various concentrations (0, 5, 10, 50 ng/ml) of recombinant human TGF-beta2 for 7 days. On day 7 viable cells were counted by trypan blue exclusion assay in a hemocytometer. The data are expressed as average ± SD from three experiments performed in triplicate.

Figure 3: TGF-beta1 suppresses proliferation of adult rodent NSCs without induction of apoptosis

Adult rodent NSC cultures were treated with 10 ng/ml of recombinant human TGF-beta1 for 7 days. For the last 24 hours 5 µM BrdU were added to the culture medium. On day 7 cells were lysed and proliferation rate was determined by measurement of BrdU incorporation into the DNA with the ELISA technique (a). Apoptosis was measured by measurement of fragmented DNA with the ELISA technique (b). The data are expressed as average ± SD from three experiments performed in triplicate.

Figure 4: The effect of TGF-beta1 on NSCs is reversible

Adult rodent neural stem and precursor cell cultures were treated with 10 ng/ml of recombinant human TGF-beta1 for 7 days. On day 7 cells were dissociated, counted by trypan blue exclusion assay and TGF-beta1 pre-treated cells were reseeded in with or without 10 ng/ml TGF-beta1. This procedure was performed every 7 days. The data are expressed as average ± SD from three experiments performed in triplicate.

Figure 5: The cloning efficacy of stem and precursor cells is not affected by TGF-beta1

Adult rodent neural stem and precursor cell cultures were dissociated and resulting single cells were used for clonal analysis by limiting dilution technique 0,5 cells/well were seeded on 96-Well plates in the presence or absence of 10 ng/ml TGF-beta1. During 6 weeks of culture each well was

manually screened for colonies using phase-contrast microscopy, and only wells that originally contained one single cell were referred to as clones.

Figure 6: TGF-beta1 is suppressing expression of TGF-betaRI and TGF-betaRII on adult rodent NSCs

Adult rodent NSC cultures were treated with 10 ng/ml of recombinant human TGF-beta1 for 7 days. On day 7 cells were lysed and total RNA was extracted. RT-PCR for several subtypes of all three TGF-beta-receptors was performed.

Figure 7: Antibodies against TGF-beta1 are incapable of blocking TGF-beta1 effects on adult rodent NSCs

Adult rodent NSC cultures were treated with 10 ng/ml of recombinant human TGF-beta1 for 7 days in the presence or absence of anti-TGF-beta antibody. On day 7 viable cells were counted by trypan blue exclusion assay in a hemocytometer. The data are expressed as average ± SD from three experiments performed in triplicate.

Figure 8: Antibodies against TGF-betaRII can reduce TGF-beta1 effects on adult rodent NSCs

Adult rodent NSC cultures were treated with 10 ng/ml of recombinant human TGF-beta1 for 7 days in the presence or absence of anti-TGF-betaRII antibody. On day 7 viable cells were counted by trypan blue exclusion assay in a hemocytometer. The data are expressed as average ± SD from three experiments performed in triplicate.

Figure 9: Soluble TGF-RII completely inhibits TGF-beta1 induced suppression of NSC proliferation

Adult rodent NSC cultures were treated with 10 ng/ml of recombinant human TGF-beta1 for 7 days in the presence or absence of soluble anti-TGF-betaRII. On day 7 viable cells were counted by trypan blue exclusion assay in a

hemocytometer. The data are expressed as average ± SD from three experiments performed in triplicate.

Figure 10: The anti-proliferative effects of TGF-beta1 on adult rodent NSCs are at least in part mediated by cell cycle arrest

(a) Adult rodent NSC cultures were treated with 10 ng/ml of recombinant human TGF-beta1 for 7 days. On day 7 cells were fixed and stained with propidium-iodide to determine the cell-cycle distribution of the cells by FACS-technique.
(b) Total RNA-extracts of cells were made at different time-points after TGF-beta1 stimulation (0, 0,5, 1, 2, 12 and 24 hours) and analyzed by RT-PCR for up-regulation of p21-mRNA.

Figure 11: TGF-beta1 treated adult rodent NSCs retian their stem cell properties and show elevated levels of ßIII-tubulin-mRNA, a neuronal marker

(a) Adult NSCs were stimulated with TGF-beta1 for 7 days. After dissociation the cells were reseeded on poly-ornithin/laminin-coated coverslips for an additional 24 hours before fixation. The cells were stained for the expression of neural stem cell markers (nestin), markers for neurons (betaII-tubulin), astrocytes (GFAP) and oligodendrocytes (GalC). (b) Total RNA-extracts of TGF-beta1-treated cells were made and analyzed by RT-PCR for expression of betaIII-tubulin-mRNA.

Figure 12: Strong expression of TGF-RII on the ependymal layer of the subventricular zone

Sections of intact adult rat brain were stained for the expression of TGF-RII, visualized using the fluorescent immuno-histocytochemistry technique and analyzed by confocal microscopy.

Figure 13: TGF-RII is expressed on stem and precursor cells in vivo

Sections of intact adult rat brain were stained for the expression of TGF-RII and Nestin, a marker for neural stem cells, visualized using the fluorescent immuno-histocytochemistry technique and analyzed for co-expression by confocal microscopy.

Figure 14: TGF-betaRII-expressing cells can be isolated using Cell sorting techniques

Dissociated adult NSCs were incubated with primary antibodies against TGF-RII and secondary antibodies coupled to paramagnetic beads. The cell suspension was magnetically sorted using the MACS-system (Miltenyi) and negative positive cells were counted by trypan blue exclusion assay in a hemocytometer. The data are expressed as average ± SD from three experiments.

Figure 15: Massive suppression of in vivo NSC proliferation by TGF-beta1 in the SVZ of adult rats

Adult female Fisher-344 rats were infused either CSF or TGF-beta1 in the lateral ventricle of the brain. 50 mg/kg bodyweight of BrdU were administered inra-peritoneally daily. After 7 days of infusion, rats were perfused, fixed and analyzed for BrdU-incorporation by immuno-histocytochemistry.

In the disorders described above, microglial cells, and potentially perivascular resting macrophages, are attracted from protein aggregates, cell debris, inflammation, inflammatory response in atherosclerosis, or acute trauma/hypoxia associated cell death. This may be an acute, subacute or chronic process. During the activation process the

activated microglial cell population (including macrophages from the vessel wall or other sources) releases a number of inflammatory cytokines into the extracellular matrix, draining either into small venules or directly into the CSF-space. These cytokines will reach the CSF-compartment and will be immediately available at all locations, which are surrounded to some extend by CSF. Among these cytokines is TGF-beta.

It was demonstrated (Monje, Toda et al. 2003) that neuroinflammation inhibits neurogenesis and that inflammatory blockade with indomethacin, a common nonsteroidal anti-inflammatory drug, restores neurogenesis after endotoxin-induced inflammation and augments neurogenesis after cranial irradiation. Monje et al. Loc.cit. define the microglia produced cytokine IL-6 as the molecule that suppresses neurogenesis upon inflammation. The prior art does not disclose TGF-beta as the main regulator down-regulating neurogenesis and neurorepair after injury or under pathological conditions. In contrast, the prior art considered TGF-beta as a neuroprotective agent preventing injured or lesioned neurons from cell death, and tried to up-regulate TGF-beta in CNS disease conditions (for review see (Unsicker and Krieglstein 2002). Zhang et al (Zhang, Hoffmann et al. 1997) demonstrate that TGF-beta has an effect on developing quail neural crest cells. Here, TGF-beta inhibited proliferation of both pluripotent neural crest cells (and/or their immediate derivatives) and of committed melanogenic cells, causing a decrease in colony size. In addition, and in contrast to the present invention, neurogenesis increased significantly in the presence of TGF-beta. The number per colony of both adrenergic cells and sensory neuron precursors increased in TGF-beta-treated neuroblast-positive colonies.

Transforming growth factor beta1 (TGF-beta1) is a

multifunctional pleiotropic cytokine with a central role in tissue repair (Grande 1997). It controls cell proliferation and banks the immune response. It belongs to a family of peptides with pleiotropic effects widely distributed throughout the body and in particular in the immune system (for review see (Letterio and Roberts 1998)). In addition to the TGF-betas, bone morphogenetic proteins (BMP) and activin make up the BMP-superfamily (Miyazono, Kusanagi et al. 2001). The three isotypic TGF-betas are extremely well conserved across species with a greater than 99% identity between the mature TGF-beta sequences of various mammalian species (Derynck, Jarrett et al. 1986; Derynck and Rhee 1987).

TGF-beta is a 24 kd protein produced by many cells, including B and T lymphocytes and activated macrophages, as well as by many other cell types. Among the effects of TGF-beta on the immune system are inhibitions of IL-2-receptor induction, IL-1-induced thymocyte proliferation and blocking of gamma interferon-induced macrophage activation. TGF-beta is believed to be involved in a variety of pathological conditions (reviewed in (Border and Ruoslahti 1992)).

TGF-beta is generally secreted as latent precursor consisting of TGF-beta non-covalently associated with a protein designated latency-associated protein (LAP; reviewed in (Harpel, Metz et al. 1992)). This latent complex requires enzymatic cleavage of carbohydrate groups or transient acidification to release the active cytokine. Purified LAP by itself binds active TGF-beta with high affinity to form a latent complex. A DNA encoding a 278 amino acid peptide corresponding to pre-pro-TGF-beta, terminating just prior to the mature form of TGF-beta and containing a Cys33 to Ser33 substitution has been expressed (Derynck, Jarrett et al. 1985) and found to bind TGF-beta and render it latent.

TGF-betas have important roles in cell growth and differentiation, organ development, matrix formation, wound repair and immune function (Letterio and Roberts 1998; Blobe, Schiemann et al. 2000). While TGF-beta is a potent growth-inhibitory substance for many cell types, it stimulates proliferation of fibroblasts and osteoblasts. It is also a potent stimulator of extracellular matrix production by fibroblasts and osteoblasts (Massague 1987; Robey, Young et al. 1987), inhibits matrix degradation and up-regulates receptors for matrix interaction. TGF-beta1 has been implicated as a key causative factor in the pathogenesis of liver fibrosis (Rockey, Housset et al. 1993; Border and Noble 1994) and at least as one crucial mediator of both the beneficial and detrimental effects of cyclosporine A on the immune system and the kidney (reviewed in (Khanna, Cairns et al. 1999)). In addition, various chronic progressive fibrotic kidney disorders in humans and experimental models have been shown to be associated with stimulation of the TGF-beta system (Bitzer, Sterzel et al. 1998).

TGF-beta regulates cellular processes by binding to three high-affinity cell-surface receptors known as types I, II and III. The type III receptors are the most abundant receptor type. They bind TFG-beta and transfer it to its signaling receptors, the type I (RI) and II(RII) receptors. Primary binding of the ligand occurs with the RII receptor, promoting formation of a heterodimer with RI and activation of signaling (for review see Hu and Zuckerman, 2001). Upon binding of a ligand to a type II receptor, type II receptor kinases phoshorylate serine and threonine residues within the intracellular GS (glycine-serine-rich) domain of type I receptors, leading to activation of the type I receptor. The activated TGF-beta R1 then interacts with an adaptor molecule SARA (Smad anchor for receptor activation) (Tsukazaki, Chiang et al. 1998), which facilitates the access of particular

members of the Smad family of proteins, called receptor-regulated Smads (R-Smads) to activated TGF-beta receptors. The activated type I receptor kinases then phosphorylate R-Smads differentially at two serine residues at their extreme C termini (summarized in (Itoh, Itoh et al. 2000)). R-Smads include Smad 1,-2,-5 and-8 proteins. Smad2 and-3 mediate the signaling of TGF-beta and activins; and Smad8 mediates the signaling of ALK-2 receptor kinases (Lagna, Hata et al. 1996; Liu, Hata et al. 1996; Zhang, Feng et al. 1996). Inhibitory Smads (I-Smads) consist of vertebrate Smad6 and Smad7 and Drosophila daughters against dpp (Dad). Unlike R-Smads, which augment the signaling of TGF-beta molecules, I-Smads inhibit TGF-beta superfamily signaling. Whereas Smad6 appears to inhibit BMP signaling preferentially, Smad7 acts as a general inhibitor of TGF-beta family signaling (Itoh, Landstrom et al. 1998; Souchelnytskyi, Nakayama et al. 1998; Ishisaki, Yamato et al. 1999). I-Smads can bind stably to the intracellular domain of activated type I receptors, thereby inhibiting further signal transduction by preventing the phosphorylation of R-Smads by the receptor (Imamura, Takase et al. 1997; Inoue, Imamura et al. 1998; Souchelnytskyi, Nakayama et al. 1998). The expression of I-Smads appears to be part of a negative feedback loop. The expression of Smad6 and-7 can be induced rapidly and in some cases directly by BMP, activin and/or TGF-beta in cultured cells. In addition, Smad3 and -4 can directly bind to the Smad7 promoter to mediate activation of this promoter by activin or TGF-beta (Nagarajan, Zhang et al. 1999). In addition to stimulation through the TGF-beta-Smad pathway, Smad7 expression can also be induced by IFN-gamma through the Jak/Stat pathway (Ulloa, Doody et al. 1999), by TNF-alpha through activation of NF-kappaB (Bitzer, von Gersdorff et al. 2000), and by norepinephrine also through NF-kappaB (Kanamaru, Yasuda et al. 2001). In addition to the function of Smad7 as an inhibitor of the phosphorylation of R-Smads by type I receptors at the cytoplasm/cell membrane

border, Smad7 was also found to occur abundantly in the nuclei of certain cells and to be exported from the nucleus upon TGF-beta stimulation or a change in cell substrate (Itoh, Landstrom et al. 1998; Zhu, Iaria et al. 1999). Pulaski (Pulaski, Landstrom et al. 2001) showed that mutation in a major phosphorylation site of Smad7 at Ser-249 did not affect the inhibitory effect of Smad7 on TGF-beta or BMP7 signaling and did not interfere with nuclear localization of Smad7. Instead, phosphorylation of Smad7 at Ser-249 was shown to be important for its ligand- independent ability to regulate transcription. Mice over-expressing Smad7 exhibit defective T cell responses to TGF-beta1, show markedly greater cytokine production in vitro, and show enhanced antigen-induced airway inflammation (Nakao, Miike et al. 2000).

TGF-beta1 down-regulates G1 and G2 cyclin-dependent kinases and cyclins in terms of both kinase activity and protein amount (Hu and Zuckerman 2001). TGF-beta1 also inhibits phosphorylation of the product of the retinoblastoma tumor suppressor gene pRb at multiple serine and threonine residues in human myeloid leukemia cells (Hu and Zuckerman 2001). The under-phosphorylated pRb associates with transcription factor E2F-4 in G1 phase, whereas the phosphorylated pRb mainly binds to E2F-1 and E2F-3. Because TGF-beta1 up-regulates p130(pRb family member)/E2F-4 complex formation and down-regulates p107(pRb family member)/E2F-4 complex formation, with E2F-4 levels remaining constant, these results suggest that E2F-4 is switched from p107 to pRb and p130 when cells exit from the cell cycle and arrest in G1 by the action of TGF-beta1. The "cdk inhibitor" p27 is both a positive and a negative regulator of TGF-beta1-mediated cell cycle control. Although TGF-beta1 has been reported to be a selected inhibitor of normal primitive hematopoietic stem cells, TGF-beta inhibits both primitive and more differentiated myeloid leukemia cell lines (for review see (Hu and Zuckerman 2001)).

Most attention was drawn on TGF-beta1's neuroprotective activity , its role in neural development and on its role in modulating immune responses. TGF-beta1 has been shown in a number of studies to be neuroprotective in vitro and in vivo (for review see (Krieglstein, Strelau et al. 2002; Unsicker and Krieglstein 2002; Dhandapani and Brann 2003)). Agonist studies have demonstrated that TGF-beta1 reduces neuronal cell death and infarct size following middle cerebral artery occlusion (MCAO), while conversely, antagonist studies have shown increased neuronal cell death and infarct size after MCAO, suggesting that TGF-beta1 has a neuroprotective role in cerebral ischemia. Recent work with adenoviral- mediated overexpression of TGF-beta1 in vivo in mice has further implicated a neuroprotective role for TGF-beta1 in cerebral ischemia, as evidenced by a reduction in neuronal cell death, infarct size, and neurological outcome. Additionally, numerous in vitro studies have documented the neuroprotective ability of TGF-beta1 in neurons from a variety of species, including rats, mice, chicks, and humans. Of significant interest, TGF-beta1 was shown to be protective against a wide variety of death-inducing agents/insults, including hypoxia/ischemia, glutamate excitotoxicity, beta-amyloid, oxidative damage, and human immunodeficiency virus. The neuroprotective effect of TGF-beta1 has been related to its ability to maintain the mitochondrial membrane potential, to stabilize Ca2+ homeostasis, to increase the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xl, to inhibit caspase-3 activation and to induce plasminogen activator inhibitor-1 (Prehn, Bindokas et al. 1994; Buisson, Nicole et al. 1998; Zhu, Ahlemeyer et al. 2001). Studies in embryonic stem cells have demonstrated a primitive neural stem cell as a component of neural lineage specification that is negatively regulated by TGF-beta-related signalling (Tropepe, Hitoshi et al. 2001). Endogenous expression of TGF-alpha, another TGF family member, has been

shown to positively regulate adult neurogenesis (Tropepe, Craig et al. 1997). TGF-alpha is necessary for the full proliferation of progenitor cells present in the subependyma and the full production of the neuronal prescursors that migrate to the olfactory bulb. In TGF-alpha knock out mice, proliferation of these progenitor cells also is diminished with age, likely because of a lengthening of the cell cycle (Tropepe, Craig et al. 1997). Senescence or the absence of endogenous TGF-alpha does not affect the numbers of neural stem cells isolated in vitro in the presence of epidermal growth factor (Tropepe, Craig et al. 1997).

The use of TGF-beta for immunomodulation in humans is severely limited by its toxicity, including excessive stimulation of matrix production, nephrotoxicity and other detrimental effects. TGF-beta has oncogenic potential and has been implicated inglomerulopathies, pulmonary fibrosis, scleroderma and chronic graft versus host disease. In addition, while TGF-beta is an extremely potent immunosuppressive cytokine, several lines of evidence indicate that chronic stimulation of TGF-beta expression-both disease-related or in transgenic animal models-can paradoxically lead to or enhance autoimmune inflammation.

There is increasing evidence that the powerful anti-inflammatory properties of TGF-beta as a negative regulator of T-cell immune response play a key role in the pathophysiology a variety of CNS pathologies (Kulkarni, Huh et al. 1993; Benveniste 1998). Therefore, this cytokine is regarded as an injury-related peptide and a potential target for therapeutic intervention (Krieglstein, Strelau et al. 2002). Neuroinflammation and microglial pathology are associated with many neurological diseases. Here, the most classical ones are clearly neuro-immunological disease such as Multiple Sclerosis. But it includes also diseases of cognition in which

memory loss features prominently, such as Alzheimer's Disease, Lewy Body Dementia, AIDS Dementia Complex, Vascular Dementia, or less prominently, such as Pick's Disease, Progressive Supranuclear Palsy, Corticobasal Degeneration, and Creutzfeldt-Jakob's Disease (Baker, Lu et al. 1999; Brew 1999; Mackenzie 2000; Dickson 2001). In addition, inflammatory programs are activated after acute lesions such as stroke, traumatic brain and spinal cord injuries (Carmichael 2003). In different animal models for Creutzfeldt-Jacob's Disease activation of microglia and up-regulation of TGF-beta1 bee reported (Baker, Lu et al. 1999).

Alzheimer's Disease

Increased expression of TGF-beta was demonstrated in brain biopsies from patients suffering from various acute or chronic neurodegenerative disorders including stroke, Parkinson's disease, or Alzheimer disease (Mattson, Barger et al. 1997; Pratt and McPherson 1997). In patients of Alzheimer's disease, the level of TGF-beta1 in plaques, cerebrospinal fluid and serum is elevated compared to controls (van der Wal, Gomez-Pinilla et al. 1993; Chao, Ala et al. 1994; Chao, Hu et al. 1994; Flanders, Lippa et al. 1995). TGF-beta is present in senile amyloid plaques found in the CNS and is overexpressed in Alzheimer's disease brain compared with controls (Finch (1993) Cell Biochem 53, 314-322). In addition, levels of TGF-beta1 mRNA correlate with the degree of cerebrovascular amyloidosis in Alzheimers's Disease and TGF-beta1 immunoreactivity in such cases is elvated along cerebral blood vessels (Wyss-Coray, Masliah et al. 1997). In transgenic mice over-expressing TGF-beta1 under control of the astrocytic promoter of the GFAP gene the elevated level of TGF-beta1 results in accumulation of basement membrane proteins in blood vessels preceeding the formation of cerebrovasuclar amyloid deposits (Wyss-Coray, Lin et al. 2000). Furthermore, it was implicated that TGF-beta1 is an important modifier of amyloid

deposition in vivo and that TGF-beta1 might promote microglial processes that inhibit the accumulation of amyloid plaques in the brain parenchyma (Wyss-Coray, Lin et al. 2001).

Parkinson's Disease

Parkinson's disease is associated with elevated levels of TGF-beta1. In brains of Parkinson's patients the levels of TGF-beta1 are increased compared to controls in affected dopaminergic striatal regions and in the ventricular cerebrospinal fluid, but not in the unaffected cerbral cortex (Mogi, Harada et al. 1995; Vawter, Dillon-Carter et al. 1996). Furthermore, TGF-beta1 has been implicated in the pathophysiology of Parkionson's disease. Overexpression of TGF-beta1 in the mesostriatal system of an MPTP animal model for Parkinson's Disease greatly decreased the number of dopaminergic neurons, suggesting that TGF-beta1 might be a risk factor for this disease.

Amyotrophic Lateral Sclerosis

Motor Neuron Disorders, as in combined MND (ALS = Amyotrophic Lateral Sclerosis) or lower motor neuron disease (SMA = spinal muscular atrophy) are characterized by spontaneous or genetically defined defects in motor neuron function in the spinal cord or/and motor cortex. As for example, in familiar ALS, mutations in the gene for Superoxid Dismutase (SOD) lead to a premature stress induced ageing of motor neurons with subsequent cell death. When 80% of motor neurons have vanished, patients become wheel chair bound from secondary muscular atrophy, have increasing swallowing, speaking and respiratory difficulties until they succumb. Onset of SMA`s is usually during childhood, in case of ALS onset is later during adulthood. In all cases, usually mild to moderate microglial activation is seen again during the course of the disease, indicating the effort of these cells to clear the debris of the dying cells. The serum and cerebrospinal fluid levels of

TGF-beta1 in ALS patients are elevated (Houi, Kobayashi et al. 2002; Ilzecka, Stelmasiak et al. 2002). Furthermore, the cerebrospinal fluid concentration of TGF-beta1 correlated with the duration of ALS (Ilzecka, Stelmasiak et al. 2002). Neuroinflammation has been postulated to be a drug target for ALS (Weydt, Weiss et al. 2002). Kriz and coworkers demonstrated that minocycline, an anti-inflammatory agent, slows down the disease progression in a transgenic mouse model for ALS (SOD1 - G37R - mouse) (Kriz, Nguyen et al. 2002).

Spinocerebellar Atrophies (SCA`s)

In this entity the neuronal elements of the cerebellum, and to different extent and depending upon the genetic defect, other CNS regions, degenerate. In principle, these cells also age and dye prematurely, and are not replaced in a timely fashion. Inflammatory programs are activated under these diseases (Evert, Vogt et al. 2001).

Creutzfeldt-Jacob's Disease

In different animal models for Creutzfeldt-Jacob's Disease activation of microglia and up-regulation of TGF-beta1 has been reported (Baker, Lu et al. 1999).

Acute ischemic/hypoxic lesions, CNS-Trauma

In an acute traumatic/hypoxic lesion of the brain parenchyma preformed latent TGF-ß will be transformed into active TGF-ß by proteases released during the trauma. This TGF-ß available at once in large quantities is mainly neuroprotective; it also prevents the parenchyma from being invaded by large quantities of inflammatory PMNC`s, that would lead to immediate death of the individual through severe brain edema. In a secondary autocrine loop (after a few hours) TGF-ß is induced in surviving neuronal and ectodermal/mesenchymal CNS-cells, leading to further neuroprotection. After a second interval TGF-ß again is induced for several days, probably to induce

glial scarring and angiogenesis. The lack of functional neurogenesis and neuroregeneration might be explained in the initial phase by high local tissue levels of TGF-beta1 in the damaged area. Later on in the process, vertricular TGF-beta1 will rise, and precursor cells are directly inhibited at the subventricular zone - regeneration will not take place in larger amounts. During the very acute phase and all later phases, infiltration of the affected brain parenchyma by hematopoetic stem cells will always be suppressed by TGF-ß.

Increased expression of TGF-beta was demonstrated in post mortem brain tissue of human stroke victims (Krupinski, Kumar et al. 1996). In vitro data support a neuroprotective role of the TGF-beta pathway with particular reference to NMDA-induced neuronal death in excitotoxic paradigms such as hypoxia-ischemia (Prehn, Backhauss et al. 1993; Buisson, Nicole et al. 1998). On the contrary, findings from in vivo-studies consistently describe induction of TGF-beta1 mRNA expression within hours after focal brain ischemia and up-regulation persisting for several weeks after the insult (Wang, Yue et al. 1995; Lehrmann, Kiefer et al. 1998; Ruocco, Nicole et al. 1999). More detailed data by Ali and coworkers (Ali, Docagne et al. 2001) localized the significantly enhanced expression of TGF-beta1 to the ischemic penumbra, i. e. to the transitional metabolic zone between the ischemic core and the peri-infarct zone. As blocking of the biological activity of TGF-beta by a specific antagonist increased both excitotoxic and ischemic lesions, data derived from rodent stroke models suggest that activation of the TGF-beta signaling pathway may be associated with neuroprotection (Ruocco, Nicole et al. 1999; Ali, Docagne et al. 2001). In vivo data from a stroke model in rat identifying the cellular source of TGF-beta1 production after focal cerebral ischemia, demonstrated early induction as well as long-term up-regulation of TGF-beta1 mRNA expression confined to activated microglia and macrophages.

Therefore, TGF-beta1 mediated functions represent an immediate and persistent response in the acute ischemic brain lesion and are involved in the phase of tissue remodeling after stroke (Lehrmann, Kiefer et al. 1998). More detailed, a biphasic expression of TGF-beta1 with a first peak at 12 hours and at 7 days after permanent MCA occlusion in the infarcted tissue has been reported, the latter most probably linked to the down-regulation of inflammatory tissue response, the induction of neoangiogenesis, and glial scar formation (Logan, Berry et al. 1994; Yamashita, Gerken et al. 1999). The up-regulation of TGF-beta1 gene expression extends from 3 hours to 4 days after transient forebrain ischemia (Zhu, 2000), up to 15 days after permanent MCA occlusion (Wang, 1995), and from 6 hours to 21 days after global brain ischemia (Lehrmann, 1995), respectively. Data from in vivo studies concerning the intraarterial or the intracerebroventricular application of TGF-beta1 showed both treatment before (Gross, 1993) and after induction of pathology (Gross,1994, McNeill, 1994) to be associated with a significant reduction of neuronal loss and infarct size in a rabbit model of thromboembolic stroke or a rat model of severe hypoxic-ischemic brain injury, respectively. In transient global ischemia in rats, Henrich-Noack and colleagues were able to show significant protection of pyramidal CA1 cells by intrahippocampal injection of TGF-beta1 prior to ischemia (Henrich-Noack, 1996). In mice over-expressing TGF-beta1 after adenoviral gene transfer Pang and coworkers (2001) demonstrated a reduction of infarct volume, associated with an inhibition of the inflammatory response to MCA occlusion in terms of reduced leukocyte and monocyte/macrophage infiltration into the ischemic brain tissue (Pang, 2001). Highly elevated levels of TGF-beta1mRNA were also reported for the ischemic penumbra in brain samples of human stroke victims (Krupinski, 1996). Furthermore, the enhanced expression of several TGF-beta isoforms and of the type I receptor protein in reactive processes surrounding

ischemic brain lesions was demonstrated in human autopsy and biopsy material (Ata, 1997). While TGF- beta1 serum levels were not significantly different in stroke patients and healthy volunteers, a close correlation between TGF-beta1 levels and both clinical and neuroradiological parameters of brain injury have been reported (Kim, 1996, Slevin, 2000, Stanzani, 2001).

Traumatic brain Injury

Experimental traumatic brain injury (TBI) results in a rapid and significant necrosis of cortical tissue at the site of injury. In the following hours and days, secondary injury exacerbates the primary damage resulting in significant tissue destruction and neurological dysfunction (Faden, 1993). Alterations in excitatory amino acids, increased oxidative stress and increased apoptosis contribute to progressive neuronal death following TBI. (summarized in Sullivan, 2002). Rimaniol et al. described a biphasic production of TGF-beta following cerebral trauma, with a first peak after 30 min. and a second peak 48h after the lesion (Rimaniol, 1995). Lindholm et al. showed increased production of TGF-beta1 mRNA in the rat cerebral cortex after a penetrating brain injury (Lindholm, 1992). In this paper they argued that TGF-beta1 expressed in the lesioned brain may play a role in nerve regeneration by stimulating nerve growth factor (NGF) production and by controlling the extent of astrocyte proliferation and scar formation. Logan et al. showed a diffuse increase of TGF-beta1 mRNA and protein around the cerebral stab wound at 1,2 and 3 days; at 7 and 14 d after lesion the distribution was more localized to the region of the glial scar (Logan, 1992). They suggested to use TGF-beta1 antagonists to limit the pathogenesis associated with matrix deposition in the CNS wound. Krieglstein et al. showed that the survival promoting effect of Glial cell line-derived neurotrophic factor (GDNF) in vivo and in vitro requires the

presence of TGF-beta (Krieglstein, 1998). In a very recent study, Peterziel et al. demonstrated that the TGF-beta induced GDNF responsiveness in neurons is caused by the TGF-beta induced recruitment of the gycosyl-phosphatidyl-inositol-anchored GDNF receptor (GFR) alpha1 to the plasma membrane (Peterziel, 2002).

Epilepsia

In experimentally-induced seizures, an up-regulation of TGF-beta1 has been demonstrated (Plata-Salaman, Ilyin et al. 2000).

Neuroinflammatory Disorders of the CNS

Acute and sub-acute inflammatory disorders of the central nervous system are frequently self-terminated. In consequence to acute bacterial or viral meningoencephalitis, however, frequently long-lasting or even permanent intellectual deficits are observed, leading to a considerable failure rate at school and further education in children or at work in adults. Chronic generalized epilepsy, in addition, is frequently a consequence of meningoencephalitis. Again, potential neuronal regeneration is probably perturbed already in the acute stage of the disease - by activated microglial cells, secreting high amounts of TGF-ß into CSF, signaling to progenitor cell associated TGF-ßR, and thereby stopping neuronal progenitor cells to invade and restore the affected brain.

CNS-Autoimmune disorders, like Multiple Sclerosis, however, are characterized by an acute phase of neuroinflammation with mainly demyelinating lesions, which may occur in bouts or as primarily progressive forms. During this relatively acute phase, a prolonged microglial activation will increasingly occurr, leading to a state of axonal/neuronal and oligodendroglial degeneration in later disease stages. These

secondary degenerating events lead to the final debilitating state of longterm-MS patients. Recent data indicate, that missing regeneration is most evident at areas near to the CSF-compartment or microglial noduli. This is probably due to a chronic activation of these microglial cells, that among other cytokines produce TGF-ß, which again will be released into the CSF and signal to neuronal precursor cells via TGF-ßR upon the latter.

Multiple Sclerosis

Acute and subacute inflammatory disorders of the central nervous system are frequently self-terminated. Autoimmune disorders like Multiple Sclerosis (MS), however, are characterized by an acute phase of neuroinflammation with mainly demyelinating lesions which may occur in bouts or as primarily progressive forms. During this relatively acute phase microglial activation will increasingly occur leading to a state of axonal/neuronal and oligodendral degeneration. Recent data indicate that missing regeneration is most evident at areas near to the CSF-compartment. These events lead to the final debilitating state of long-term MS-patients.

The endogenous TGF-beta-production was shown to be up-regulated in the CNS of experimentally induced MS, the experimental autoimmune encephalitis (EAE) model, and presumably plays a down-modulatory role during the recovery phase of acute EAE (Khoury, 1992; Racke, 1992 ; Issazadeh, 1995; Issazadeh, 1998). While TGF-beta expression was described to be up-regulated in the CNS of Lewis rats during the remission phase of (monophasic) EAE, a significant expression of regulatory cytokines such as TGF-beta (andIL-4 andIL-10) was not found in the DA rat CNS or lymphoid tissues at various time points (Issazadeh, 1996). Cytokine analysis demonstrated that the mRNA expression ofIL-10 and TGF-beta1 was generally low in both acute EAE and the first attack of

chronic EAE and upr egulated at later stages of chronic EAE. It was suggested that anti-inflammatory cytokines play only a minor role in the relapse (Tanuma, 2000). Recovery of disease in mice transgenic for an MBP-specific T cell receptor induced to develop EAE was associated with an immune deviation of Th1 T cells towards cells that secreted IL-4, IL-10, and TGF-beta both in the periphery and in the CNS [Chen, 1998]. Kiefer and colleagues carried out a systematic study of TGF-beta expression (Kiefer, 1998). In actively induced monophasic EAE in the Lewis-rat, in situ hybridization revealed strong expression of TGF-beta1 in meningeal and perivascular mononuclear infiltrates at onset of the disease, continued expression in perivascular infiltrates and scattered mononuclear cells at maximal disease severity, and expression in scattered parenchymal cells during recovery. Cellular expression of TGF-beta1 by T-cells, macrophages, and microglia summed up to a long-lasting elevation of TGF-beta1mRNA extending well into the recovery phase. While TGF-beta1 expressed early in the disease by T-cells was thought to contribute to inflammatory lesion development, its expression by microglial cells was suggested to potentially contribute to recovery (Kiefer, 1998).

TGF-beta1 and 2 mRNA-expression in CNS tissue from MS cases, as demonstrated by in situ hybridization, was found mainly in perivascular rather than parenchymal cells, suggesting circulating inflammatory cells as the major source (Woodroofe, 1993). In summary, they found both a stronger expression and a differently localized cellular distribution in MS (active demyelinating and chronic active and inactive lesions) as opposed to control tissue. In a bioassay from peripheral blood cultures, TGF-beta like activity was found to be increased in patients with active disease as opposed to those with inactive disease and healthy donors and was found in particular in the subgroup tested during the regression of symptoms (Beck,

1991). Decreased TGF-beta production by lymphocytes of patients with MS correlated directly with disease activity. MS patients with active disease produced less TGF-beta than MS patients with stable disease. The cells producing TGF-beta were primarily CD8+ T cells and CD45RA+T cells (Mokhtarian, 1994). Using a semi-quantitative PCR the expression of TGF-beta and IL-10 was reported to decrease prior to a relapse while the expression of TNF-alpha and lymphotoxin increased (Rieckmann, 1995). In an open-label phase 1 trial of 11 patients with secondary progressive (SP) MS the safety of recombinant active TGF-beta2 was assessed (Calabresi, 1998).

In chronic psychiatric disorders like Schizophrenia probably another basic mechanism will result in a similar situation of chronic neuronal degeneration, resulting in severe dementia: Owing to information processing deficits of so far unknown origin, chronic neuronal under-usage is observed. Obviously, this also finally leads to cell death and debris in larger amounts that has to be cleared by microglial cells. Microglial activation takes place, and the combination of neuronal under-usage with TGF-ß secretion probably prevents repair, leading to chronic dementia. Also in affective disorders, like Major Depression, deafferentiation of the serotoninergic system of the brainstem raphe will lead to local neuronal degeneration, microglial activation and secondary inhibition of neuronal repair.

Arteriosclerosis / Atherosclerosis

In recent publications it is getting increasingly clear that inflammatory processes are probably the major driving force in atherosclerosis, of course also within the CNS. Especially in patients with so called microangiopathic dementia (Binswanger`s Disease, Leukoaraiosis) , a rapid decline in cognitive function is observed during the disease. Severe macrophage and microglial activation occurs during

atherosclerotic changes of the wessel wall. Interestingly, in these patients, severe demyelination occurs directly in the afflicted white matter, exactly where the small perforating arteries normally supply oxygen and nutrition to the parenchyma. The demyelination of the white matter leads to severe defects in cortical cognitive association, later also to cortical atrophy, probably from deafferentiation. Demyelination as the origin of dementia in this group of patients, is clearly explained by the suppression of neuronal precursor cells to become oligodendrocytes and produce enough myelin in the white matter, its again a failure of repair.

Retinal and cochlear degeneration

Retinal and cochlear degenerative disorders are widespread, as e.g. Macular Degeneration or Cochlear Deafness. In these disorders similar mechanisms occurr as in primary CNS degeneration. In these disorders, however, the CSF is replaced either by the endolymphatic fluid (cochlea) or by the vitreous (retina).

Neurogenesis

Until recently, the general consensus was that, unlike many other organs, the central nervous system (CNS) has no capacity for spontaneous regeneration and cell replacement. This view has been changed by numerous studies demonstrating that specialized regions exist within the adult brain capable of continuous cell replacement. Recent studies on neurogenesis, the development of neurons from immature precursor cells, suggest that it might be possible to replace degenerated or dead neurons through this process. Support for the therapeutic potential of endogenous neurogenesis includes evidence that neurogenesis continues to occur in the brains of adult mammals, including rodents, primates and humans (Kuhn (1996) J Neurosci 16(6): 2027-33; Eriksson (1998) Nat Med, 4(11): 1313-

1317) (Eriksson, Perfilieva et al. 1998; Gould, Tanapat et al. 1998; Gould, Reeves et al. 1999; Kornack and Rakic 1999; Kornack and Rakic 2001). The cellular substrates of neurogenesis are multipotent neural stem cells that reside in the neurogenic regions. They are characterized by i) their potential to proliferate, ii) to self renew and iii) to generate lineage restricted and fate determined, proliferative precursor cells that upon and during differentiation mature and generate new neurons, astrocytes and oligodendrocytes, the three major cell types of the CNS. Therefore, neurogenesis requires proliferation, cell fate commitment, neuronal lineage restriction and subsequent differentiation and maturation. Neural stem cells are slowly dividing multipotent cells residing in neurogenic regions. They give rise to fast dividing neuronal determined precursor cells that by proliferation increase and potentiate the neurogenic activity of a neural stem cell. After several rounds of division they turn postmitotic and start to neuronally differentiate and to mature. Dynamic changes and regulation of the level of neurogenesis occur mainly at the level of neural stem cell and neuronal precursor cell proliferation.

In the adult brain, two neurogenic regions constantly generate new neurons. The dentate gyrus of the hippocampus (Altman and Das 1965; Kaplan and Hinds 1977) contains neural stem cells in the subgranular layer. These cells divide, and generate a pool of neuronal precursors that migrate into the hippocampal granule cell layer (Cameron, Woolley et al. 1993). There, the cells differentiate into functional neurons that integrate into the hippocampal circuitry (Markakis and Gage 1999; van Praag, Schinder et al. 2002). The function of hippocampal neurogenesis is still under debate, but correlative data suggest, that neurogenesis might be involved in learning and memory that involves the hippocampus, in particularly in hippocampal memory clearance (Jaffard and Meunier 1993)

(Gould, Beylin et al. 1999) (Feng, Rampon et al. 2001). In additon, it was suggested that neurogenesis might be involved in prychiatric disorders, such as depression (Kempermann 2002; Kempermann 2002). The second neurogenic area of the adult brain is the subventricular zone (SVZ) of the lateral wall of the lateral ventricles. This structure originates during from the embryonic ventricular zone, which is the mitotic active germinal zone of the developing CNS. It retains the proliferative and neurogenic capacity throughout life (Gates, Thomas et al. 1995; Hauke, Ackermann et al. 1995; Kuhn, Winkler et al. 1997). The SVZ contains neural stem cells that proliferate and give rise to proliferating neuronally determined precursors. These precursors leave the SVZ to migrate along the rostramigratory stream (RMS) rostral into the olfactory bulb (OB), where they differentiate into granule cell neurons and periglomerular neurons and integrate into the neuronal circuitry (Lois and Alvarez-Buylla 1993; Luskin 1993; Lois and Alvarez-Buylla 1994) (Carlen, Cassidy et al. 2002). SVZ neurogenesis is not restricted to rodents, but can also be found in primates and humans (Kaplan 1983; McDermott and Lantos 1991; Eriksson, Perfilieva et al. 1998; Gould, Reeves et al. 1999; Bernier, Bedard et al. 2002). Although neurogenesis still takes place in the adult mammalian CNS, the damaged brain is largely incapable of functionally significant structural self-repair. Two strategies can be envisaged to overcome these limitations: the stimulation of endogenous neurogenesis two generate more new neurons and the ex vivo propagation of neural stem cells and transplantation of the cells.

Regulation of Neurogenesis

Neurogenesis underlies dynamic changes and is regulated at different levels. Hippocampal neurogenesis is up-regulated by global factors such as enriched environment (Kempermann, Kuhn et al. 1997), physical activity (van Praag, Christie et al.

1999), dietary restriction (Lee, Seroogy et al. 2002), growth factors such as FGF-2 (Kuhn, Winkler et al. 1997), IGF-1 (Aberg, Aberg et al. 2000), VEGF (Jin, Zhu et al. 2002), erythropoetin (Shingo, Sorokan et al. 2001), molecules such as Sildenafil (Viagra) (Zhang, Wang et al. 2002), anti-dpressants (Malberg, Eisch et al. 2000), rolipram (Nakagawa, Kim et al. 2002), and lesions such as transient global ischemia (Liu, Solway et al. 1998), seizure (Parent, Yu et al. 1997), traumatic brain injury (Dash, Mach et al. 2001). Vice versa, hippocampal neurogenesis is down-regulated by cytokines such as IL-6 (Vallieres, Campbell et al. 2002), by molecules such as corticosterone (Karishma and Herbert 2002), PCPA (Brezun and Daszuta 1999), and nicotine (Abrous, Adriani et al. 2002). Neurogenesis in the SVZ/RMS/OB axis is up-regulated by enriched odor exposure (Rochefort, Gheusi et al. 2002), FGF-2 (Kuhn, Winkler et al. 1997), BDNF (Pencea, Bingaman et al. 2001), and Erythropoietin (Shingo, Sorokan et al. 2001), and down-regulated by EGF (Kuhn, Winkler et al. 1997) or by anti-erythropoietin antibody treatment (Shingo, Sorokan et al. 2001). The notion that enhanced neurogenesis might be beneficial in the context of neurological diseases is strengthened by the fact that up-regulated neurogenesis is accompanied by functional improvement after hypoxic/ischemic lesion. After transient occlusion of the MCA newly formed neurons can be identified in the neostriatum (Parent, 2002; Arvidson, 2002). Exogenous factors, such as EGF and bFGF increase the level of neurogenesis even in non-neurogenic regions after global ischemia (Nakatomi, 2002). Furthermore, as a prerequisite for function brain repair by endogenous neurogenesis, it was demonstrated that after lesions newly born neuronal precursors migrate towards the lesion site (Fallon, Reid et al. 2000; Magavi, Leavitt et al. 2000; Arvidsson, Collin et al. 2002).

Neural stem cells in vitro and Transplantation

An alternative for stimulation of endogenous neurogenesis is transplantation of neural stem cells or progeny thereof. These require a critical mass of cell generated in vitro. Different sources of cells, such as dissociated fetal mesencephalic tissue, in vitro expanded stem cells derived from blastocysts or embryonic forebrains and neural stem cells from the adult brain have been investigated for their potential use in transplantation experiments, see, inter alia, Brundin (2000) Brain 123: 1380-90; Freed (2001) N Engl J Med 344: 710-9; Bjorklund (2002) Proc Natl Acad Sci U S A 99: 2344-2349; Brustle (1996) Curr Opin Neurobiol 6: 688-95; Englund (2002) Exp Neurol. 173: 1-21 or Gage (2000) Science 287: 1433-8. In humans suffering of Parkinson's disease, implantation of fetal tissue has resulted in some degree of functional recovery (Piccini (1999) Nat Neurosci 2: 1137-40; Brundin, 2000, loc. cit., or Freed, 2001 loc.cit.). However, the highly limited access to fetal tissue and the ethical concerns surrounding its use in patients have strengthened the search for alternative neuronal-restricted precursor cell sources, in particular those harvested from adult tissues. Neural stem cells can be isolated from adult brains and expanded in vitro (Gage (1995) ProcNatlAcad Sci USA 92 (25):11879-11883) (Wachs, Couillard-Despres et al. 2003). These cells can subsequently be transplanted into the brain, where they are able to migrate extensively and to differentiate into neurons and glia (Suhonen et al. (1996) Nature, 1996.383 (6601): 624-627). Neural precursor cells transplanted into local regions of the brain can differentiate into neurons, and have the potential to improve symptoms in disorders like Parkinson's disease (Herman, et al. (1992) Curr Opin Neurobiol, 2 (5): 683-689;Bjorldund (1992) Curr Opin Neurobiol, 2 (5): 683-689; Gage (2000)Science, 287 (5457): 1433-1438). However, there are several disadvantages of intracerebral neural precursor cell transplants. First, surgical transplantation may result in

local tissue damage or stroke. Second, large numbers of neural precursors from fetal tissues are difficult to obtain. Third, the use of precursors from certain sources, such as human embryos, is ethically and politically controversial. Fourth, neural degeneration in some CNS diseases is extensive, multifocal or even global, which may require widespread replacement beyond the capabilities of surgical transplantation. Finally, intracerebral transplantation may be associated with adverse effects related to the unregulated function of ectopic tissue (Freed et al.(2001) NEngl J Med, 344 (10): 710-719).

Neurogenesis and Changes by Disease

Alterations in neurogenesis have been demonstrated in neurological and psychiatric diseases. Acute lesions, such as stroke, seem to induce, at least transiently, an up-regulation of neurogenesis in rodents. After transient occlusion of the MCA newly formed neurons can be identified in the neostriatum (Parent, 2002; Arvidson, 2002). In addition, exogenous factors, such as EGF and bFGF increase the level of neurogenesis even in non-neurogenic regions after global ischemia (Nakatomi, 2002). Furthermore, as a prerequisite for function brain repair by endogenous neurogenesis, it was demonstrated that after lesions newly born neuronal precursors migrate towards the lesion site (Fallon, Reid et al. 2000; Magavi, Leavitt et al. 2000; Arvidsson, Collin et al. 2002). In chronic degenerative disorders of the CNS, such as Alzheimer's Disease, and in psychiatric disorders, such as depression, neurogenesis is down-regulated, and it is speculated that impaired neurogenesis results in the manifestation of these disorders. In two different animal models of Alzheimer's Disease, presenelin-1 deficient mouse and mutant APP over-expressing mice, adult neurogenesis is impaired (Feng, Rampon et al. 2001) (Haughey, Nath et al. 2002). A causative role of neurogenesis in the manifestation

of depression was postulated (Kempermann and Kronenberg 2003), but still lacks substantial evidence.

Neurogenesis and Changes by Inflammation

The brain is an immuno-privileged tissue that exhibits dampened adaptive immunity in response to injury, lesion, degenerative processes, infection, or tumor formation. However, the brain does exhibit a robust innate immune response mediated by microglia. Microglia are cells in the CNS that defend against invading microorganisms and clean up by engulfing the debris of dying cells. In addition, the inflammatory mediators released by microglia during an innate immue response strongly influence neurons and ability of process information (Bezzi, Domercq et al. 2001). Inflammation seems to be detrimental for neurogenesis. LPS-induced inflammation in the brains of adult rats impairs hippocampal neurogenesis and blocking the activation of microglia by minocycline boosts neurogenesis. (Ekdahl, Claasen et al. 2003). Pro-inflammatory mediators released by microglia seem to be important contributors to the block in hippocampal neurogenesis. In vivo, the number of activated microglia correlates negatively with the production of new neurons (Monje, Toda et al. 2003). Neuronal determination and differentiation in vitro, as measured by the fraction of DCX (a marker for young neurons) expressing cells, is inhibited when NSCs are exposed to activated, but not quiescent, microglia (Monje, Toda et al. 2003). The cytokine IL-6, but not IL-1beta, not TNF-alpha, and not INF-gamma, is apparently the key regulator of this inhibition, since addition of an antibody that blocks IL-6 to NSCs in vitro abolishes the inhibitory effects of activated microglia in NSCs in vitro. The effect is specific for neurogenesis, since gliogenesis is not affected. The data from Monje 2003 loc.cit. demonstrate that the microglia and IL-6 activity results in a decreased accumulation of new neurons, most likely due to reduced

neuronal differentiation rather the selective changes in the proliferation or death of neuroblasts or immature neurons. IL-6 and its downstream JAK-STAT signaling pathway have been implicated in the selective differentiation of cerebral cortical precursors into astrocytes (Bonni, Sun et al. 1997). Inhibition of neurogenesis by IL-6 might be due to increased production of astrocytes at the expense of neuronal precursors, particularly as astrocytes and neuronal precursors seem to share a common stem cell. Alternatively, inhibition of neurogenesis by IL-6 may be a consequence of a decrease in neuronal progenitor cell proliferation or an increase in the number of these cells undergoing apoptosis. Alterations in the microenvironemt of NSCs may allow ectopic neurogenesis to occur (Magavi, Leavitt et al. 2000; Nakatomi, Kuriu et al. 2002), or on the other hand reduce or block neurogenesis resulting in learning and memory deficits (Cameron, Tanapat et al. 1998; Monje, Mizumatsu et al. 2002; Madsen, Kristjansen et al. 2003).

Influencing the levels of TGF-beta1

(a)Up-Regulation of TGF-beta1 and its effects

Many studies tried to increase TGF-beta1 levels for neuroprotective or immunoregulatory purposes. Agonist studies have demonstrated that TGF-beta1 reduces neuronal cell death and infarct size following middle cerebral artery occlusion (MCAO), while conversely, antagonist studies have shown increased neuronal cell death and infarct size after MCAO, suggesting that TGF-beta1 has a neuroprotective role in cerebral ischemia. Recent work with adenoviral-mediated overexpression of TGF-beta1 in vivo in mice has further implicated a neuroprotective role for TGF-beta1 in cerebral ischemia, as evidenced by a reduction in neuronal cell death, infarct size, and neurological outcome. Additionally, numerous in vitro studies have documented the neuroprotective ability

of TGF-beta1 in neurons from a variety of species, including rats, mice, chicks, and humans. Of significant interest, TGF-beta1 was shown to be protective against a wide variety of death-inducing agents/insults, including hypoxia/ischemia, glutamate excitotoxicity, beta-amyloid, oxidative damage, and human immunodeficiency virus. The neuroprotective effect of TGF-beta1 has been related to its ability to maintain the mitochondrial membrane potential, to stabilize Ca2+ homeostasis, to increase the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xl, to inhibit caspase-3 activation and to induce plasminogen activator inhibitor-1 (Prehn, Bindokas et al. 1994; Buisson, Nicole et al. 1998; Zhu, Ahlemeyer et al. 2001).

The use of TGF-beta for immunomodulation in humans is severely limited by its toxicity, including excessive stimulation of matrix production, nephrotoxicity and other detrimental effects. TGF-beta has oncogenic potential and has been implicated inglomerulopathies, pulmonary fibrosis, scleroderma and chronic graft versus host disease. In addition, while TGF-beta is an extremely potent immunosuppressive cytokine, several lines of evidence indicate that chronic stimulation of TGF-beta expression-both disease-related or in transgenic animal models-can paradoxically lead to or enhance autoimmune inflammation.

Mice genetically targeted to overexpress bioactive TGF-beta1 specifically within astrocytes were reported to show a phenotype with severe CNS pathology at high levels of expression. While unmanipulated heterozygous transgenic mice from a low expressor line showed no such alterations, increasing TGF-beta 1 expression in this line by injury-induced astroglial activation or generation of homozygous offspring did result in the abnormal phenotype (Wyss-Coray, 95). Astroglial over-expression of TGF-beta 1 was not

associated with obvious CNS infiltration by hematogenous cells (Wyss-Coray, 1995). However, these mice were more susceptible to EAE-induction with earlier and more severe CNS inflammation. Thus, local expression of TGF-beta 1 within the CNS parenchyma can enhance immune cell infiltration and intensify the CNS impairment resulting from peripherally triggered autoimmune responses (Wyss-Coray 1997). An Alzheimer's disease-like pathology with perivascular astrocytosis and deposition of amyloid in cerebral blood vessels was observed in older mice expressing low-levels of transgenic active TGF-beta (Wyss-Coray, 2000).

Kiefer (1998), analyzing TGF-beta expression in monophasic EAE of the Lewis rat, found evidence for early expression in T cells, possibly contributing to inflammatory lesion development while the later occurring expression within microglia was suggested to play a down-modulatory role. When Ag-specific murine T cell lines and clones were cultured in the presence of TGF-beta the effector function of these autoreactive cells and demyelinating lesion formation upon adoptive transfer in experimental autoimmune encephalomyelitis were markedly enhanced (Weinberg1992). In another EAE model it was shown that the effects of TGF-beta on autoimmune disease expression vary depending on the timing of treatment with respect to disease induction. Daily i. p. injections of 0. 2-2ug TGF-beta 1 or TGF-beta 2 on days 5 to 9 after immunization were highly protective, while injections on days 1-5 or 9-13 were not. TGF-beta treatment on days 5-9 prevented the accumulation of T cells in brain and spinal cord, as assayed on days 15 to 20. Anti-TGF-beta accelerated and aggrevated EAE when administered on days 5 and 9, but not on day 12. It was concluded that the protective effect of TGF-beta is exerted at the level of the target organ, CNS and/or its vascular endothelium and that there was a small window of 4 days in which TGF-beta exerts its protective effect (Santambrogio,

1993).

Various strategies that were successful in modulating EAE and suggested TGF-beta as part of the protective effect proved not to be effective or showed considerable toxicity in clinical trials. In an open-label phase 1 trial of 11 patients with secondary progressive (SP) MS the safety of recombinant active TGF-beta2 was assessed (Calabresi, 1998). Groups of patients were treated in a dose-escalation scheme with 0, 2 ug/kg, 0, 6 ug/kg or 2,0 pg/kg. Treatment was administered i. v. , three times weekly for four weeks unless discontinued earlier. A reversible decline in the glomerular filtration rate developed in five patients (three with 0, 6 pg/kg, both with 2,0 ug/kg), transient mild to moderate anemia in seven, hypertension in two and a maculopathy in one patient. The nephrotoxicity and anemia were likely to be related to TGF-beta-treatment. A beneficial effect or an effect on clinical or imaging parameters was not observed (Calabresi, 1998).

These indications of systemic side-effects considerably lessened the interest in TGF-beta as a therapeutic tool for MS (Calabresi 1998; Wiendl 2000). In addition, in a phase III clinical trial of oral myelin tolerization in RRMS neither clinical nor MRI outcome parameters were significantly different between myelin and placebo- treated patients (Panitch 97, Francis 97). However, it has also been shown that it is considerably more difficult to treat ongoing EAE by mucosal tolerization (discussion in Xu 2000) or TGF-beta itself (discussion in Thorbecke 2000) than to prevent disease. Administration of a neutralizing anti-TGF-beta-antibody resulted in the prevention of renal failure, excess matrix gene expression and glomerular mesangial matrix expansion in db/db diabetic mice (Ziyadeh, 2000).

Chronic TGF-beta up-regulation plays a central role in

progressive matrix accumulation and renal insufficiency observed in diabetic nephropathy (reviewed in Sharma and McGowan, 2000). The pathology of systemic multidose administration of recombinant human TGF-beta1 in rats and rabbits was described by Terrell (1993): A 14 day pilot study was performed in rats using rhTGF-beta1 produced in human A293 cells. After administration of 1000pg/kg i. v. two rats died after 5 days. The remaining rats were sacrificed at that point. The mid-dose and low-dose-groups received 100g/kg and 10pg/kg i. v. daily for 14 days, respectively. Adverse events were most striking in the high-dose group but qualitatively similar changes were seen at the mid-dose level albeit less severe and delayed in onset. Besides certain histo-pathological changes, the rats displayed reduced body weight (from day 3) and an increased hematocrit on day 3 with a subsequent decrease. In the discussion of their findings Terrell and associates stated that the relative severity and rapidity with which some of the observed changes-both clinical and histo-pathological such as the hepatic involution and the enostosis-occurred in the high-dose preparations was remarkable (Terrell, 1993).

(b) TGF-beta1 blocker

Soluble forms of TGF-beta receptors will bind TGF-beta and prevent binding to membrane-associated TGF-beta receptors. TGF-beta receptors are described by Wang et al. (Cell 67:797-805, 1991) and Lin et al. (Cell 68:775-785, 1992). Soluble forms of TGF-beta receptors may be prepared by methods that are known in the art. For example, deletion mutants lacking the transmembrane domain of a TGF-beta receptor can be prepared, which will express a soluble TGF-beta binding protein. Miyazono et al. (Adv. Immunol. 55:181; 1994) have reviewed TGF-beta receptors.

Other types of TGF-beta antagonists are also known in the art. For example, Yamaguchi et al. (Nature 346:281-284, 1990) discuss decorin, a small chondroitin-dermatan sulphate proteoglycan that binds TGF-beta and modulates the activity of this growth factor. Ohtsuki and Massague (Mol. Cell. Biol. 12:261-265, 1992) disclose protein kinase inhibitors that block certain biological activities of TGF-beta. T. cruzi produces a cysteine protease (cruzain or cruzipain; Eakin et al., J. Biol. Chem. 267:7411; 1992) which converts inactive TGF-beta precursor into active, mature TGF-beta. The design and use of protease inhibitors as drugs is well known in the art (Design of Enzyme Inhibitors as Drugs; Sandler and Smith, eds; 1989, Oxford University Press; Proteinase Inhibitors Medical and Biological Aspects; Katunuma, Umezawa and Holzer, eds., 1983, Springer-Verlag); thus, inhibitors of cruzain can be prepared and will be useful as TGF-beta antagonists.

Thus, the present invention relates to a compound interfering with (a) the biological activity of TGF-beta1 or its expression or (b) the TGF-beta1/TGF-R signaling, for the preparation of a pharmaceutical composition for the prevention or treatment of a disease, wherein neurogenesis or neuroregeneration has a beneficial effect.

The term "interfering" as used herein means modulating, preferably reducing or eliminating, the biological activity of TGF-βbeta1 or its expression, or TGF-beta1/TGF-R signaling. The modulation of the biological activity can be effected by direct interaction or binding of a compound to TGF-beta1 and/or TGF-R, preferably, TGF-R_{II} or by indirect interaction, e.g., by interacting with a compound that is associated with the biological activity of TGF-beta1 or TGF-R.

Suitable compounds acting as agents targeting TGF-beta1, -2, -3 or TGF-beta$R_{I, II, III}$, or its signal transduction to interfere with this regulatory circuit with the aim to improve neuroregeneration or increase neuronal/hematopoetic stem cell or precursor cell recruitment to the CNS, including all types of local or systemic transplantation (e.g. ex vivo propagation, allogeneic cells) are listed below. This applies not only for treatment (be it acute, chronic, local, systemic) but also for all diagnostic (e.g. in preparing for other treatments) and prognostic (be it socioeconomic, intended treatment, insurance, or other) purposes:

(a) Plasmids, vectors or natural/synthetic/mutated viruses, oligonucleotides of various types of modification (e.g. PTO, LNA, 2`F-ANA, protein-nucleotide complexes, RNA_i, $_{si}$RNA or mikro $_{mi}$RNA, Methylmetoxy-, Phosphoroamidates, PNA, Morpholino, Phosphoramidate, Cyclohexen (CeNA), gap-meres, ribozymes, aptamers, CpG-oligos, DNA-zymes, riboswitches, or lipids or lipid containing molecules,
(b) peptides, peptide complexes, including all types of linkers,
(c) small molecules, modifyers of rafts or caveoli,
(d) modifyers of golgi apparatus,
(e) antibodies and their derivatives, especially chimeras, Fab-fragments, Fc-fragments, or
(f) carriers, liposomes, nanoparticles, complexes, or any other delivery systems containing the above named constructs,
can be used to target the above mentioned circuit to restore or improve neuroregeneration.

Further compounds suitable for the purposes of the present invention and methods how to identify/select such compounds are in more detail described below.

Preferably, in a pharmaceutical composition, such compound as described above is combined with a pharmaceutically acceptable carrier. "Pharmaceutically acceptable" is meant to encompass any carrier, which does not interfere with the effectiveness of the biological activity of the active ingredient and that is not toxic to the host to which it is administered. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc.. Such carriers can be formulated by conventional methods and the active compound can be administered to the subject at an effective dose.

An "effective dose" refers to an amount of the active ingredient that is sufficient to affect the course and the severity of the disease, leading to the reduction or remission of such pathology. An "effective dose" useful for treating and/or preventing these diseases or disorders may be determined using methods known to one skilled in the art (see for example, Fingl et al., The Pharmocological Basis of Therapeutics, Goodman and Gilman, eds. Macmillan Publishing Co., New York, pp. 1-46 ((1975)).

Administration of the suitable compositions may be effected by different ways, e.g. by intravenous, intraperetoneal, subcutaneous, intramuscular, topical or intradermal administration. The route of administration, of course, depends on the kind of therapy and the kind of compound contained in the pharmaceutical composition. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends on many factors, including the patient's size, body surface area, age, sex, the particular compound to be administered, time and route of

administration, the kind of therapy, general health and other drugs being administered concurrently.

In a preferred embodiment of the present invention, the disease that can be prevented/treated is a neurodegenerative disorder, a neuroinflammatory disorder of the CNS, an acute ischemic brain lesion or hypoxic brain lesion. Preferred examples of neurodegenerative disorders are Alzheimer's Disease, Parkinson's Disease, Creutzfeldt Jakobs Disease (CJD), Hallervorden Spatz Disease and Huntington's Disease. A preferred example of a neuroinflammatory disorder is Multiple Sclerosis (MS).

The person skilled in the art can easily identify or generate compounds useful for the treatments of the present invention based on the knowledge of the amino acid sequence of TGF-β1 and its receptors and the nucleotide sequences of the genes encoding these proteins; see Derynck et al., 1985; Lin et al; 1992.

In a further preferred embodiment of the present invention, the compound useful for interfering with the expression of the gene encoding TGF-β1 or TGF-R, preferably TGF-R_{II}, is an antisense oligonucleotide.

The generation of suitable antisense oligonucleotides includes determination of a site or sites within the TGF-β1 gene or TGF-R for the antisense interaction to occur such that the desired effect, e.g., inhibition of expression of the protein, will result. A preferred intragenic site is (a) the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene or (b) a region of the mRNA which is a "loop" or "bulge", i.e., not part of a secondary structure. Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently

complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect. In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound does not need to be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., in the case of therapeutic treatment.

The skilled person can generate antisense compounds according to the present invention on the basis of the known DNA sequences for TGF-β1 and TGF-R, respectively.

"Oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases. While antisense oligonucleotides are a preferred form of the antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention comprise from about 8 to about 50 nucleobases (i.e. from about 8 to about 50 linked nucleosides). Particularly preferred antisense compounds are antisense oligonucleotides, even more preferably those comprising from about 15 to about 25 nucleobases. Antisense compounds include ribozymes, external guide sequences (EGS), oligonucleotides (oligozymes), and other short catalytic RNAs or catalytic oligonucleotides which hybridize to the target nucleic acid and inhibit its expression.

Alternatively, the pharmaceutical composition of the invention contains a vector allowing to transcribe an antisense oligonucleotide of the invention, e.g., in a mammalian host. Preferably, such a vector is a vector useful for gene therapy. Preferred vectors useful for gene therapy are viral vectors, e.g. adenovirus, herpes virus, vaccinia, or, more preferably, an RNA virus such as a retrovirus. Even more preferably, the retroviral vector is a derivative of a murine or avian

retrovirus. Examples of such retroviral vectors which can be used in the present invention are: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV) and Rous sarcoma virus (RSV). Most preferably, a non-human primate retroviral vector is employed, such as the gibbon ape leukemia virus (GaLV), providing a broader host range compared to murine vectors. Since recombinant retroviruses are defective, assistance is required in order to produce infectious particles. Such assistance can be provided, e.g., by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus under the control of regulatory sequences within the LTR. Suitable helper cell lines are well known to those skilled in the art. Said vectors can additionally contain a gene encoding a selectable marker so that the transduced cells can be identified. Moreover, the retroviral vectors can be modified in such a way that they become target specific. This can be achieved, e.g., by inserting a polynucleotide encoding a sugar, a glycolipid, or a protein, preferably an antibody. Those skilled in the art know additional methods for generating target specific vectors. Further suitable vectors and methods for in vitro- or in vivo-gene therapy are described in the literature and are known to the persons skilled in the art; see, e.g., WO 94/29469 or WO 97/00957.

In order to achieve expression only in the target organ, e.g., brain tissue, the DNA sequences for transcription of the antisense oligonucleotides can be linked to a tissue specific promoter and used for gene therapy. Such promoters are well known to those skilled in the art (see e.g. Zimmermann et al., (1994) Neuron 12, 11-24; Vidal et al.; (1990) EMBO J. 9, 833-840; Mayford et al., (1995), Cell 81, 891-904; Pinkert et al., (1987) Genes & Dev. 1, 268-76).

Within an oligonucleotide structure, the phosphate groups are

commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage. Specific examples of preferred antisense compounds useful in the present invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. Oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. Modified oligonucleotide backbones which can result in increased stability are known to the person skilled in the art, preferably such modification is a phosphorothioate linkage.

A preferred oligonucleotide mimetic is an oligonucleotide mimetic that has been shown to have excellent hybridization properties, and is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone (see, e.g., Nielsen et al., Science 254 (1991), 1497-1500.)

Modified oligonucleotides may also contain one or more substituted or modified sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. A particularly preferred modified sugar moiety is a 2'-O-methoxyethyl sugar moiety.

Oligonucleotides of the invention may also include nucleobase modifications or substitutions. Modified nucleobases include

other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine etc., with 5-methylcytosine substitutions being preferred since these modifications have been shown to increase nucleic acid duplex stability.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include lipid moieties such as a cholesterol moiety, cholic acid, a thioether, a thiocholesterol, an aliphatic chain, e.g., dodecandiol or undecyl residues, a phospholipid, a polyamine or a polyethylene glycol chain, or adamantane acetic acid, a palmityl moiety, or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety.

The present invention also includes antisense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation

of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers.

In a further preferred embodiment of the present invention, the compound useful for interfering with the biological activity of TGF-β1 is a compound reducing or inhibiting the binding of TGF-β1 to its receptor.

Preferred examples of such compounds are (neutralizing) antibodies directed against TGF-β1 or a TGF-β receptor; see Lin et al., 1992, preferably the TGF-β receptor II. The term „antibody", preferably, relates to antibodies which consist essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody preparations. Monoclonal antibodies are made from an antigen containing, e.g., a fragment of TGF-β1 or a corresponding receptor by methods well known to those skilled in the art (see, e.g., Köhler et al., Nature 256 (1975), 495). As used herein, the term „antibody" (Ab) or „monoclonal antibody" (Mab) is meant to include intact molecules as well as antibody fragments (such as, for example, Fab and F(ab')2 fragments) which are capable of specifically binding to protein. Fab and F(ab')2 fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact

antibody. (Wahl et al., J. Nucl. Med. 24: 316-325 (1983)). Thus, these fragments are preferred, as well as the products of a FAB or other immunoglobulin expression library. Moreover, antibodies useful for the purposes of the present invention include chimerical, single chain, and humanized antibodies.

Further preferred compounds for the use of the present invention are soluble TGF-β receptors. The term "soluble" as used herein in the context of receptors preferably relates to fragments of the receptor only comprising the extracellular domain(s) of the receptor or a part thereof which can still bind its natural ligand, e.g., TGF-β1. The person skilled in the art can determine such fragments based on the known amino acid sequences of the receptors and the determination of the extracellular domain of the receptors can be carried out by use of well known methods, e.g., by computer programs (hydrophilicity plot).

In a particular preferred embodiment of the use of the present invention, said soluble TGF-β receptor is the TGF-β receptor II.

The present invention also relates to a method for identifying a compound interfering with (a) the biological activity of TGF-β1 or its expression, or (b) the TGF-β1/TGF-R signaling, comprising the steps of:

(a) incubating a candidate compound with a test system comprising TGF-β1 and neuronal precursor cells; and

(b) assaying the expression of active TGF receptors or the proliferation of the neuronal precursor cells;

wherein

(c) an abolition of (i) the suppression of expression of active TGF receptors or (ii) suppression of proliferation of the neuronal precursor cells compared to the test system in the absence of said test compound is indicative

of the presence of a candidate compound having the desired properties.

Examples of such candidate molecules include antibodies, oligonucleotides, proteins (e.g., receptors), or small molecules. Such molecules can be rationally designed using known techniques.

Preferably, said test system used for screening comprises substances of similar chemical and/or physical properties, most preferably said substances are identical. The compounds which can be prepared and identified according to a use of the present invention may be expression libraries, e.g., cDNA expression libraries, peptides, proteins, nucleic acids, antibodies, small organic compounds, ligands, hormones, peptidomimetics, PNAs or the like.

More recently, WO 98/25146 described further methods for screening libraries of complexes for compounds having a desired property, especially, the capacity to agonize, bind to, or antagonize a polypeptide or its cellular receptor. The complexes in such libraries comprise a compound under test, a tag recording at least one step in synthesis of the compound, and a tether susceptible to modification by a reporter molecule. Modification of the tether is used to signify that a complex contains a compound having a desired property. The tag can be decoded to reveal at least one step in the synthesis of such a compound. Other methods for identifying compounds which interact with a TGF-β1 according to the invention or nucleic acid molecules encoding such molecules are, for example, the in vitro screening with the phage display system as well as filter binding assays or "real time" measuring of interaction using, for example, the BIAcore apparatus (Pharmacia).

All these methods can be used in accordance with the present invention to identify a compound interfering with the biological activity of TGF-β1 or its expression, or TGF-β1/TGF-R signaling.

It is also well known to the person skilled in the art, that it is possible to design, synthesize and evaluate mimetics of small organic compounds that, for example, can act as a substrate or ligand to a TGF-β receptor. For example, it has been described that D-glucose mimetics of hapalosin exhibited similar efficiency as hapalosin in antagonizing multidrug resistance assistance-associated protein in cytotoxicity; see Dinh, J. Med. Chem. 41 (1998), 981-987.

The gene encoding TGF-β1 or TGF-R can also serve as a target for screening inhibitors. Inhibitors may comprise, for example, proteins that bind to the mRNA of the gene encoding TGF-β1 or TGF-R, preferably TGF-R_{II}, thereby destabilizing the native conformation of the mRNA and hampering transcription and/or translation. Furthermore, methods are described in the literature for identifying nucleic acid molecules such as an RNA fragment that mimics the structure of a defined or undefined target RNA molecule to which a compound binds inside of a cell resulting in retardation of cell growth or cell death; see, e.g., WO 98/18947 and references cited therein. These nucleic acid molecules can be used for identifying unknown compounds of pharmaceutical interest, and for identifying unknown RNA targets for use in treating a disease. These methods and compositions can be used for identifying compounds useful to reduce expression levels of TGF-β1 and/or the corresponding receptor(s).

The compounds which can be tested and identified according to the method of the invention may be expression libraries, e.g., cDNA expression libraries, peptides, proteins, nucleic acids,

antibodies, small organic compounds, hormones, peptidomimetics, PNAs or the like (Milner, Nature Medicine 1 (1995), 879-880; Hupp, Cell 83 (1995), 237-245; Gibbs, Cell 79 (1994), 193-198 and references cited supra). Furthermore, genes encoding a putative regulator of TGF-β1 and/or which excert their effects up- or downstream of TGF-β1 may be identified using, for example, insertion mutagenesis using, for example, gene targeting vectors known in the art. Said compounds can also be functional derivatives or analogues of known inhibitors. Such useful compounds can be for example transacting factors which bind to TGF-β1 or regulatory sequences of the gene encoding it. Identification of transacting factors can be carried out using standard methods in the art. To determine whether a protein binds to the protein itself or regulatory sequences, standard native gel-shift analyses can be carried out. In order to identify a transacting factor which binds to the protein or regulatory sequence, the protein or regulatory sequence can be used as an affinity reagent in standard protein purification methods, or as a probe for screening an expression library. The identification of nucleic acid molecules which encode polypeptides which interact with TGF-β1 can also be achieved, for example, as described in Scofield (Science 274 (1996), 2063-2065) by use of the so-called yeast "two-hybrid system". In this system TGF-β1 is linked to the DNA-binding domain of the GAL4 transcription factor. A yeast strain expressing this fusion polypeptide and comprising a lacZ reporter gene driven by an appropriate promoter, which is recognized by the GAL4 transcription factor, is transformed with a library of cDNAs which will express plant proteins or peptides thereof fused to an activation domain. Thus, if a peptide encoded by one of the cDNAs is able to interact with the fusion peptide comprising a peptide of, e.g., TGF-β1, the complex is able to direct expression of the reporter gene. In this way, e.g., TGF-β1 and the gene encoding TGF-β1 can be used to identify peptides and

proteins interacting with TGF-β1. It is apparent to the person skilled in the art that this and similar systems may then further be exploited for the identification of inhibitors.

Finally, the present invention relates to the use of a compound identified by the method described above for the preparation of a pharmaceutical composition for the prevention or treatment of a disease, wherein neurogenesis or neuroregeneration has a beneficial effect.

The below example explains the invention in more detail.

Example 1

TGF-β1 inhibits proliferation of adult rodent neural stem and precursor cells

Adult female mice (various strains) or Fischer-344 rats (3-4 months; Charles River, Germany) are killed, and brains and spinal cords are removed and put in 4°C DPBS (PAN, Germany) with 4.5 gm/L glucose (Merck, Germany) (DPBS/glu). Overlying meninges and blood vessels are removed. Hippocampus and ependymal zones, including subependymal and subventricular zones from the lateral wall of the lateral ventricle (SVZ), are aseptically removed. The dissected tissue is transferred to fresh DPBS/glu, washed once, transferred to Petri dishes, and dissociated mechanically. The cell suspension is washed in DPBS/glu to rinse off excess blood and resuspended in PPD solution containing 0.01% papain (Worthington Biochemicals, England), 0.1% dispase II (Boehringer Mannheim, Mannheim, Germany), 0.01% DNase I (Worthington Biochemicals), and 12.4 mM $MgSO_4$ in HBSS (PAN) without Mg_2/Ca_2 (PAA, Germany) and digested for 30 to 40 minutes at room temperature. The cell suspension is triturated every 10 minutes. Dissociated cells are collected and resuspended in serum-free DMEM/F12 medium

(Gibco, BRL, Germany) containing 2 mM L-glutamine and 0.1 gm/L penicillin/streptomycin and washed three times with accurate trituration. Finally the single-cell suspension is resuspended in NB medium (Gibco BRL, Germany) supplemented with B27 (Gibco BRL) (NB/B27), 2 mM L-glutamine (PAN), 0.1 gm/L penicillin/ streptomycin (PAN), 2 ml (0.28 U/ml) heparin (Sigma, Taufkirchen, Germany), 20 ng/ml bFGF-2 (R&D Systems, Germany), and 20 ng/ml EGF (R&D Systems, Germany). Viable cells are counted by trypan blue exclusion assay in a hemocytometer. Cells are seeded in T-25 culture flasks and cultures are maintained at 37° C in an incubator with 5% CO_2. Single cells begin to form spheres within 5 to 7 days of suspension culture and continue to grow in mass and number during the next weeks. Half of the medium is changed every 7 days. Cells from passage numbers 3 to 20 are used for the experiments (Wachs et al., 2003). The cultures of neural stem and precursor cells are further referred to as NSC's. For the dissociation process, the culture medium containing floating neurospheres is collected in a 15-ml centrifuge tube and centrifuged at 120 rcf for 5 minutes. The pellet is resuspended in 200 µl of Accutase (Innovative Cell Technologies Inc., distributed by PAA) and triturated approximately 10 times using a pipette. Then, the cell suspension is incubated at 37° C for 10 minutes. Dissociated spheres are again triturated and resuspended in 800 µl of NB/B27 medium. Dissociated cells are centrifuged at 120 rcf for 5 minutes and resuspended in NB/B27 medium (Gibco, BRL, Germany). An aliquot is counted by trypan blue exclusion assay in a hemocytometer to determine the amount of viable cells. Cells (10^5) are plated in T75 culture flasks for long-term passaging (10 ml of culture medium per flask) in NB/B27 medium. The cells obtained after Accutase treatment of primary neurospheres proliferate and yield secondary neurospheres. Secondary neurospheres are passaged 7 to 9 days after plating primary neurosphere cells. Similar to primary cultures and primary neurospheres, single cells

obtained after dissociation of secondary neurospheres proliferate and yield tertiary neurospheres (Wachs et al., 2003).

10^4 NSC's are seeded in 12-well plates in NB/B27 medium in a volume of 1 ml and grow for 7 days. 2 hours, 3 days and 6 days after seeding the cells are stimulated by addition of various concentrations (0, 2,5, 5, 10, 50 and 100 ng/ml) of recombinant human Transforming Growth Factor ß1 (TGF-ß1) (R&D Systems, Germany). On day 7, the cultures are dissociated by the use of Accutase™ and viable cells are counted by trypan blue exclusion assay in a hemocytometer. *In vitro* TGF-ß1 inhibits the proliferation of adult neural stem and precursor cells in a dose dependant manner (Figure 1). Even though the diameter of both, stimulated and unstimulated, does not change, the diameter of neurospheres cultured in the presence of TGF-ß1 is significantly reduced when compared to cultures without TGF-ß1.

Example 2

TGF-ß2 does not interfere with proliferation of adult rodent neural stem and precursor cells

The experiments are performed as described in Example 1. In contrast treatment of adult rat NSC cultures with various concentrations (0, 5, 10 and 50 ng/ml) of recombinant human Transforming Growth Factor ß2 (TGF-ß2) (R&D Systems, Germany) has no growth-inhibitory effect. Co-incubation of adult rat neural stem and precursor cell cultures with 10 ng/ml TGF-ß1 and 10 ng/ml TGF-ß2 does not reduce the TGF-ß1-induced decrease in proliferation (Figure 2).

Example 3

TGF-ß1 suppresses proliferation of adult rodent neural stem and precursor cells without induction of apoptosis

To prove, that TGF-ß1 in fact suppresses the proliferation of adult rat neural stem and precursor cell cultures, NSC cultures as described in Example 1 are additionally incubated with 5 µM BrdU (Sigma) for the last 24 hours before counting takes place. When the cells are lysed the proliferation rate is determined by measurement of BrdU incorporation into the DNA with the ELISA technique developed in our lab. DNA of cultured NSC's is isolated using the DNeasy Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol. The genomic DNA is denaturated and degraded by treatment with 0,25 M NaOH for 30 min., 0,25 HCl and 0,2 M K_2HPO_4/KH_2PO_4 pH 7. The DNA (200 ng/ml) is added on DNA-binding ELISA plates (Costar, Germany) that are precoated with 50 mM Na_2PO_4, pH 8,5, 1 mM EDTA. After an overnight incubation at 4 °C, plates are washed three times with PBS and incubated with 3% BSA/PBS for 30 min. After three times PBS washing, plates are incubated with mouse anti-BrdU Antibody (Roche, Germany, dilution 1:500) for one hour at room temperature. Following three washes in PBS, the secondary peroxidase-conjugated donkey anti-mouse antibody (Jackson Immuno Research, diluted 1:1000) is added to the wells and incubated for one hour. After three washes in PBS, plates are incubated with o-Phenylenediamine (OPD) dihydrochloride (Sigma). The colour readout of the assay is allowed to develop for 60 min. Measurements are performed at 450 nm with a microplate reader (Molecular Devices). As shown in figure 3a TGF-ß1-treated NSC's show significantly reduced BrdU-incorporation into the DNA, indicating reduction of proliferation. To show that this decrease is really due to an inhibition of proliferation and not caused by the induction of cell death, the cell lysates are tested for DNA fragments, which are only present in apoptotic cells. Apoptosis are detected by measuring cytoplasmic histone-associated DNA

fragments (mono- and oligonucleosomes) using a photometric enzyme immunoassay according to the protocol of the manufacturer (Cell Death Detection ELISA, Roche Diagnostics). In fact, TGF-ß1 does not increase apoptosis (Figure 3b), indicating a true reduction of proliferation.

Example 4

The effect of TGF-ß1 on neural stem and precursor cells is reversible

To determine, if the TGF-ß1 induced growth-inhibition is a reversible effect, NSC's are stimulated with 10 ng/ml TGF-ß1 for 7 days according to the protocol described in Example 1. After dissociation, viable cells are counted by trypan blue exclusion assay in a hemocytometer and 10^4 growth factor-stimulated NSC's are reseeded and cultured with or without 10 ng/ml TGF-ß1 according to the protocol described in Example 1. This dissociation/counting/reseeding procedure is performed every 7 days. As shown in Figure 4, after 3 weeks of culture the proliferation rate of initially TGF-ß1-treated cells now grown without TGF-ß1 returns to normal when compared to formerly untreated cells. This indicates that the effect of TGF-ß1 on adult neural stem and precursor cells is reversible. Long term incubation with TGF-ß1 does not further decrease cell proliferation.

Example 5

The cloning efficacy of stem and precursor cells is not affected by TGF-ß1

Neural stem cells have to fulfil three major requirements: i) they must be able to proliferate, ii) they must be able to self-renew, and iii) they must be able to differentiate into

all three neural cell types namely neurons, astrocytes and oligodendrocytes. As we are able to show, that TGF-ß1 is of great importance for the proliferative capacities of adult neural stem cells, we next address to the self-renewal abilities of these cells under the influence of TGF-ß1. There are several possibilities TGF-ß1 may interfere with this requirement: i) TGF-ß1 may extinguish proliferative NSC's from the cultures or, ii) TGF-ß1 may downregulate the process of proliferation in NSC's. To study which of the hypothetic effects occure in our model, we analyze the cloning efficacy of our cultured NSC's. Unstimulated seven-day-old neurospheres of low passage number are dissociated by the use of Accutase™ as described in Example 1, and the resulting single-cell suspension was used for clonal analysis. Viable cells are counted by trypan blue exclusion assay in a hemocytometer. Single cells are transferred to 96-well plates either by limited dilution technique or by FACS sorting. For limited dilution experiments, single cells are plated at a density of 0,5 cells/well of 96-well plates. For FACS-sorting experiments, individual cells were sorted according to their typical forward-scatter/side-scatter characteristics at a density of 1 cell/well in 96-well plates in 200 µl of growth medium. Half of the plates are stimulated with 10 ng/ml TGF-ß1. After the first week, half of the media is changed; then the media change is performed weekly. Each well is manually screened for colonies using phase contrast microscopy, and only wells that originally contain one single cell are referred to as clones. After 6 weeks of culture (unstimulated cells) and 10 weeks of culture (TGF-ß1-treated cells), individual clones are used to establish clonal cell lines by dissociating the clonal neurospheres and replating the single cells under the same culture conditions as described in Example 1. Cells are grown for additional passages and then analyzed for their differentiation potential. Comparison of unstimulated versus TGF-ß1 stimulated cloning assyas reveales

no differences in the ability to produce cells with the ability to self-renew (Figure 5). All clones of either treated and untreated cells have the ability to differentiate into all three neural cell types namely neurons, astrocytes and oligodendrocytes. Identical results are received, when NSC's pre-treated with TGF-ß1 undergo clonal analysis. These results indicate, that may rather downregulate the process of proliferation, than extinguish proliferative NSC's from the cultures.

Example 6

TGF-ß1 is suppressing expression of TGF-ßR$_I$ and TGF-ßR$_{II}$ on adult rodent NSC's.

In other cell types, e.g. neuroendocrine tumours of the gastroenteropancreatic tract, it is described, that TGF-ß1 may have autocrine, self-regulatory effect. To investigate if TGF-ß1 also has a autocrine effect, self-stimulatory or self-inhibitory, we analyze NSC's for the expression pattern of all three types of TGF-ß-receptors. Total RNA-extracts of both, untreated and TGF-ß1-treated adult rat NSC's are prepared using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Two µg of total RNA are reverse transcribed using Retroscript™ RT-PCR Kit (Ambion Inc.) according to the instruction manual. PCR for TGF-ßR$_I$, TGF-ßR$_{II}$, TGF-ßR$_{III}$ and GAPDH is performed with Taq PCR Master Mix (Qiagen) in an Eppendorf Master Cycler Gradient using one step 94°C, 2 min., 35 cycles (94°C, 45 sec.; 51°C (TGF-ßR$_I$) or 56°C (TGF-ßR$_{II}$) or 60°C (TGF-ßR$_{III}$) or 57°C (GAPDH), 1 min.; 72°C, 2 min.) and one final extension step (72°C, 8 min.). Forward and reverse primers used were (5`-3`): forward CTGAAATCGACCTAATTCC and reverse CCATGCTCATGATAATCC for TGF-ßR$_I$, forward CAACAACAT(AGC)AACCACAATAC and reverse ATCTTTCACTTCTCCCACAGC for TGF-ßR$_{II}$, forward TTCTACAGCTCCAAGAGAGTGC and reverse

GGAGTAGATGTACCACAAGGCC for TGF-ßR$_{III}$ and forward GGTCGGTGTGAACGGATTTG and reverse GTGAGCCCCAGCCTTCTCCAT for GAPDH.

The mRNA levels of TGF-ßR$_I$ and TGF-ßR$_{II}$ decrease upon stimulation with TGF-ß1, while TGF-ßR$_{III}$ is not expressed by NSC's (Figure 6).

Example 7

Antibodies against TGF-ß1 are incapable of blocking TGF-ß1 effects on adult rodent NSC's

In order to test, if we are able to inhibit the effects induced by TGF-ß1-treatment, several methods are investigated for their blocking efficacy. Unstimulated seven-day-old neurospheres of low passage number are dissociated by the use of Accutase™ as described in Example 1, and the resulting single-cell suspension was used for blocking analysis. Adult rodent NSC's were seeded at a density of 10^4 cells in 12-well plates in NB/B27 medium in a volume of 1 ml. 2 hours after seeding and 1 hour prior to stimulation with 10 ng/ml TGF-ß1, various concentrations of neutralizing anti-TGF-ß antibodies (R&D Systems, Germany) were added to the culture medium. To assure, that the antibody concentrations used are able to inhibit TGF-ß1, anti-TGF-ß antibodies were pre-incubated with 10 ng/ml TGF-ß1 for 1 h at room temperature before addition to the cells. 3 days and 6 days after seeding the cells are re-stimulated by addition of anti-TGF-ß antibodies and TGF-ß1 identical to the procedure performed on day 1. On day 7 the cultures are dissociated by the use of Accutase™ and viable cells are counted by trypan blue exclusion assay in a hemocytometer. Although the anti-TGF-ß antibodies are able to neutralize bioactive TGF-ß1 in the highest concentrations used (10 µg/ml) (data not shown), the antibodies do not seem to

block the TGF-ß1-induced reduction of proliferation (Figure 7).

Example 8

Antibodies against TGF-ßR$_{II}$ can reduce TGF-ß1 effects on adult rodent NSC's

In order to test, if we are able to inhibit the effects induced by TGF-ß1-treatment by applying neutralizing antibodies against TGF-ßR$_{II}$. Unstimulated seven-day-old neurospheres of low passage number are dissociated by the use of Accutase™ as described in Example 1. The resulting single-cell suspension was used for blocking analysis. Adult rodent NSC's were seeded at a density of 10^4 cells in 12-well plates in NB/B27 medium in a volume of 1 ml. 2 hours after seeding and 1 hour prior to stimulation with 10 ng/ml TGF-ß1, various concentrations of neutralizing anti-TGF-ßR$_{II}$ antibodies (R&D Systems, Germany) were added to the culture medium. 3 days and 6 days after seeding the cells are re-stimulated by addition of anti-TGF-ßR$_{II}$ antibodies and TGF-ß1 identical to the procedure performed on day 1. On day 7 the cultures are dissociated by the use of Accutase™ and viable cells are counted by trypan blue exclusion assay in a hemocytometer. Interestingly, addition of the anti-TGF-ßR$_{II}$ antibodies itself reduces proliferation of NSC's. Antibodies against TGF-ßR$_{II}$ are only able to partly inhibit TGF-ß1-induced effects even in the highest concentrations used (10 µg/ml) (Figure 8).

Example 9

Soluble TGF-R$_{II}$ completely inhibits TGF-ß1 induced suppression of NSC proliferation

A third method investigated for their blocking efficacy concerning TGF-ß1 mediated growth arrest of adult rodent NSC's represents the use of recombinant soluble TGF-ßR$_{II}$. According

to the manufacturers protocol (R&D Systems, Germany), a DNA sequence encoding the 159 amino acid residue extracellular domain of human TGF-ßR$_{II}$ (Lin et al., 1992) was fused to the Fc region of human IgG1 and the chimeric protein was expressed in a mouse myeloma cell line NSO. Unstimulated seven-day-old neurospheres of low passage number are dissociated by the use of Accutase™ as described in example 1. The resulting single-cell suspension was used for blocking analysis. Adult rodent NSC's were seeded at a density of 10^4 cells in 12-well plates in NB/B27 medium in a volume of 1 ml. 2 hours after seeding and 1 hour prior to stimulation with 10 ng/ml TGF-ß1, various concentrations of bioactive soluble recombinant human TGF-ßsR$_{II}$/Fc Chimera (R&D Systems, Germany) were added to the culture medium. 3 days and 6 days after seeding the cells are re-stimulated by addition of TGF-ßsR$_{II}$/Fc Chimera and TGF-ß1 identical to the procedure performed on day 1. On day 7 the cultures are dissociated by the use of Accutase™ and viable cells are counted by trypan blue exclusion assay in a hemocytometer. Interestingly, addition of the TGF-ßsR$_{II}$/Fc Chimera are able to completely block TGF-ß1-induced effects in a dose dependant manner(data not shown). Clearly, abrogation of active TGF-ß1 in the cell culture supernatant by pre-administration of a soluble recombinant human TGF-ßsR$_{II}$/Fc Chimera (soluble TGF-ßR$_{II}$) completely blocks TGF-ß1-induced growth-suppression of adult neural stem and precursor cells (Figure 9).

Example 10

The anti-proliferative effects of TGF-ß1 on adult rodent neural stem and precursor cells are at least in part mediated by cell cycle arrest

Characterization of the physiologic mechanisms by which adult NSC's are maintained in a quiescent state, provides

interesting information on the control of normal or pathological cell cycling. It is described for hematopoietic stem cells, that concentrations as low as 10 pg/ml of active TGF-ß1 can specifically inhibit the more primitive stem/progenitor cells (Fortunel et al., 2000). Adult rodent NSC's were seeded at a density of 10^4 cells in 12-well plates in NB/B27 medium in a volume of 1 ml and are stimulated with 10 ng/ml TGF-ß1 for 7 days according to the protocol described in Example 1. After dissociation on day 7, viable cells are counted by trypan blue exclusion assay in a hemocytometer. For cell cycle analysis, the cells are washed once with PBS, and the pellet is resuspended into 5 ml of ice-cold 70% ethanol and kept overnight at -20°C. The day after, cells are washed twice with PBS and resuspended into 1 mL of PBS and 1 mL of 0,192 M Na_2HPO_4, 4 µM citric acid, at pH 7,8. After 5 minutes of incubation at room temperature, cells are washed and resuspended into 1 mL of PBS containing 10 µg/mL RNase A. After 30 minutes of incubation at room temperature 10 µg/mL propidium iodide are added and the samples are analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Germany). Data were processed for cell cycle analysis using the WinMDI 2.8 software (J. Trotter, USA). In fact, we are able to determine a shift towards the G0/1-phase in TGF-ß1-incubated cells. This shift is consistent with a decrease in the number of cells in the S- and G2/M-phase of the cell cycle as shown by propidium-iodide staining followed by FACS-analysis (Figure 10a).

It has also been shown for hematopoietic stem cells, that there is a direct correlation between p21 expression and quiescence or slow cell cycling in response to TGF-ß1. It has been shown, that the expression of p21 is under endogenous control of TGF-ß1 and can be modulated by TGF-ß1 (Ducos et al., 2000). To investigate a potential involvement of p21 in the cell cycle shift of adult neural stem and precursor cells, we prepare total RNA-extracts according to the method

described in example 6 at different time-points after TGF-ß1 stimulation (0, 0,5 , 1, 2, 12 and 24 hours). RT-PCR for upregulation of p21-mRNA was performed according to the protocol described in example 6, with an annealing temperature of 57°C. Forward and reverse primers used were (5`-3`): forward TCCGATCCTGGTGATGTCC and reverse CGAACACGCTCCCAGACGT for p21. In fact, a slight upregulation of p21-mRNA is detectable within the first hour after stimulation (Figure 10b).

Example 11

TGF-ß1 treated adult rodent neural stem and precursor cells remain their stem cell properties and show elevated levels of ßIII-tubulin-mRNA, a neuronal marker

As the ability to differentiate into all three neural cell types - neurons, astrocytes and oligodendrocytes - is one of the requirements for neural stem cells, we investigate the differentiation-potential of adult neural stem and progenitor cells after TGF-ß1-treatment. Adult rodent NSC's were seeded at a density of 10^4 cells in 12-well plates in NB/B27 medium in a volume of 1 ml and are stimulated with 10 ng/ml TGF-ß1 for 7 days according to the protocol described in example 1. After dissociation on day 7, viable cells are counted by trypan blue exclusion assay in a hemocytometer. Single-cell suspensions are plated in the above-described media on poly-ornithine (250 µg/ml)- and laminin (5 µg/ml)-coated glass coverslips in 12-well plates at a cell density of 10^5 cells/well and ml and grown for 24 hours. In some experiments, neurospheres are plated on coated coverslips and grown under differentiation-promoting conditions (growth factor withdrawal and 1% FCS) for 7 days. Cells are fixed with phosphate buffered 4% prewarmed paraformaldehyde (37° C, pH 7,4) (4% w/v paraformaldehyde, 100 mM NaH_2PO_4, 0,4 mM $CaCl_2$, 50 mM sucrose) for 30 minutes and,

processed for immunohistochemistry. Samples are blocked for a minimum of 1 hour in fish skin gelatin buffer (0,1 M Tris-HCl, pH 7,5, 0,15 M NaCl, 1% BSA, 0,2% Teleostean gelatin (Sigma), 0,1% Triton X-100) at room temperature. The specimens are incubated overnight at 4°C with the primary antibodies at the following dilutions: mouse anti-GalC 1:500 (Chemicon, Temecula, California); rabbit anti- GFAP 1:1000 (Dako, Denmark); rabbit anti-nestin 1:200 (Chemicon, USA); mouse anti-nestin 1:200 (PharMingen International, San Diego, California); mouse anti-ßIII-tubulin 1:500 (clone 5G8; Promega, Madison, Wisconsin). The secondary fluorochrome-conjugated antibodies are diluted 1:500 (donkey anti-mouse or rabbit; Dianova, Germany). All antibody dilutions and washes are performed with the fish skin gelatin buffer. Nuclear counterstaining is performed with 4',6'-diamidino-2-phenylindole dihydrochloride hydrate at 0.25 µg/µl (DAPI; Sigma). After the last wash, the samples are briefly rinsed with PBS and mounted on slides using Prolong (Molecular Probes, The Netherlands). In cases in which antigens are sensitive to detergents (GalC), Triton X-100 is omitted from the fish skin gelatine buffer.

Undifferentiated TGF-ß1-treated cells still express nestin as a marker for neural stem cells. These cells were also able to differentiate into neurons, as shown by the expression of ßIII-tubulin, astrocytes as shown by the expression of GFAP and oligodendrocytes, as shown by the expression of GalC. (Figure 11a). Total RNA-extracts of the cells are made according to the methods described under example 6 and analyzed by RT-PCR for the expression of ßIII-tubulin-mRNA. (Primers siehe Sebastien, ebenso annealing temp., cycles 35. In fact, a slight upregulation of ßIII-tubulin-mRNA is detectable after TGF-ß1-stimulation stimulation (Figure 11b), suggesting that TGF-ß1 might have neuroprotective effects on adult rodent NSC's.

Example 12

Strong expression of TGF-R_{II} on the ependymal layer of the subventricular zone

In vivo, NSC's reside mainly in two regions of the adult mammalian brain that are known to be sites of neurogenesis: the hippocampus and the ependymal zones, including subependymal and subventricular zones from the lateral wall of the lateral ventricle. It is also from these regions that adult neural stem cell were first isolated and propagated in vitro. We analyze slices of the intact adult rat brain for the presence of the TGF-ß-receptor system. using the fluorescent immunohistochemistry technique. Brains are cryosectioned and series of every 6th section (240-µm interval) of both hemispheres from each brain are analyzed. Sections are stained for immunofluorescence using a primary antibody against TGF-ßR_{II} (R&D Systems, Germany) in a concentration of 10 µg/ml. According to the primary antibody the following secondary antibodiy is applied: anti-goat Alexa 568 (Molecular Probes, USA) Nuclear counterstain was performed with TOPRO-3 (Molecular Probes, USA). Cells were analyzed using confocal microscopy.

Figure 12 demonstrates a very strong expression of TGF-ßR_{II} on the cells of the ependymal layer, whereas the surrounding tissue only weakly stains for TGF-ßR_{II}.

Example 13

TGF-R_{II} is expressed on stem and precursor cells *in vivo*.

To further identify the cells expressing high levels of TGF-ßR_{II} we next used slices of an intact adult brain of a transgenic nestin-EGFP-mouse. In this mouse every cell expressing nestin can be detected by its green

autofluorescence under UV light. Sections of this mouse are stained for both, the expression of TGF-ßR$_{II}$ and nestin. Brains are cryosectioned and series of every 6th section (240-µm interval) of both hemispheres from each brain are analyzed. Sections are stained for immunofluorescence using a primary antibody against TGF-ßR$_{II}$ (R&D Systems, Germany) in a concentration of 10µg/ml. According to the primary antibody the following secondary antibodiy is applied: anti-goat Alexa 568. Nuclear counterstain was performed with TOPRO-3. Cells were analyzed using confocal microscopy.

Figure 13 demonstrates a very strong expression of TGF-ßR$_{II}$ on the cells of the ependymal layer, whereas the surrounding tissue only weakly stains for TGF-ßR$_{II}$. The expression of the neural stem cell marker nestin with EGFP (green channel) directly correlates with the expression of TGF-ßR$_{II}$, indicating a co-expression of these two molecules on neural stem and precursor cells *in vivo*.

Example 14

TGF-ßR$_{II}$-expressing cells can be isolated using Cell sorting techniques

One of the small disadvantages of the technique for the isolation of neural stem and precursor cells represents the insufficient capabilities identify the stem cells in order to obtain pure cultures. So far, the molecule nestin is the only reliable marker for early neural stem cells. Unfortunately this molecule is unsuitable for isolation techniques because of its intracellular localization. To investigate the possibility of isolating pure neural stem and precursor cell populations based on the expression of defined surface markers, we isolate neural stem and precursor cells due to the expression of the TGF-ßR$_{II}$ by different techniques. It is

possible to isolate TGF-ßR$_{II}$-expressing neural stem and precursor cells with two techniques: i) FACS-sorting (data not shown), and ii) MACS-sorting. Dissociated adult neural stem and precursor cells are incubated with 10 µg/ml of primary antibodies against TGF-R$_{II}$ (R&D Systems, Germany) for 20 min at room temperature. After 1 washing step with PBS the cells are incubated with the secondary antibody rabbit-anti-goat-PE (1:500) (Dianova). After 1 washing step with PBS the cells are stained with tertiary antibodies against PE coupled to paramagnetic beads according to the manufacturers protocol (Miltenyi Biotech, Germany). The cell suspension is magnetically sorted using the MACS-system according to the manufacturers protocol (Miltenyi Biotech, Germany) and negative and positive cells after sorting are counted and taken in culture (Figure 14). Approximately 20% of all sorted cells stained positive for TGF-ßR$_{II}$.

Example 15

Massive suppression of *in vivo* stem and precursor cell proliferation by TGF-ß1 in the SVZ of adult rats

As described in examples 12 and 13 TGF-ßR$_{II}$ is almost exclusively expressed on NSC's in the adult rodent brain. Therefore, we investigate, if neural stem and precursor cells in the intact brain are also susceptible to TGF-ß1 we infuse various concentrations of TGF-ß1 in the lateral ventricle of adult female Fischer-344 rats. Stainless steel cannulas, which are connected to osmotic minipumps (Model 2001, Alza) are implanted into two month-old male Fischer-344 rats (n=13) according to a previously established protocol. The animals receive either 10 ng/ml, 100 ng/ml or 500 ng/ml recombinant human TGF-ß1 (each group n=3) in or aCSF, artificial cerebrospinal fluid, (n=3) at a flow rate of 0,5 µl/hour for 7 days. During the last 6 days of the pump period, animals

receive daily intraperitoneal injections of bromodeoxyuridine (BrdU, 50 mg/kg, Sigma). After 7 days of intracerebroventricular infusion, all animals are intracardially perfused with 4% paraformaldehyde. Immunohistochemistry was performed as described before. A series of every 6th section was stained using antibodies against BrdU (Roche, 1:500). The massive suppression of neural stem and precursor cell proliferation in the SVZ and the hippocampus by TGF-ß1 could be reconfirmed as shown in figure 15.

Example 16

Massive induction of *in vivo* stem and precursor cell proliferation by recombinant human TGF-ßsR_{II}/Fc Chimera in the SVZ of adult rats

To prove, if depletion of endogenous TGF-ß1 results in an increase of neural stem and precursor cell proliferation, we infused recombinant human TGF-ßsR_{II}/Fc Chimera in the lateral ventricle of adult female Fischer-344 rats. Stainless steel cannulas, which are connected to osmotic minipumps (Model 2001, Alza) are implanted into two month-old male Fischer-344 rats (n=13) according to a previously established protocol. The animals receive either 1000 ng/ml recombinant human TGF-ßsR_{II}/Fc Chimera in aCSF (n=16) or aCSF, artificial cerebrospinal fluid, (n=16) at a flow rate of 0,5 µl/hour for 7 days. During the last 6 days of the pump period, animals receive daily intraperitoneal injections of bromodeoxyuridine (BrdU, 50 mg/kg, Sigma). After 7 days of intracerebroventricular infusion, 8 animals per group are intracardially perfused with 4% paraformaldehyde. The remaining animals have the pumps removed and are perfused after an additional four-week period without growth factor infusion.

Immunohistochemistry was performed as described above. A series of every 6th section was stained using antibodies against BrdU (Roche, 1:500). The massive induction of neural stem and precursor cell proliferation in the SVZ and the hippocampus by TGF-ß1 could be reconfirmed as shown in Figure 16.

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Claims

1. Use of a compound interfering with (a) the biological activity of TGF-β1 or its expression, or (b) the TGF-β1/TGF-R signaling, for the preparation of a pharmaceutical composition for the prevention or treatment of a disease, wherein neurogenesis or neuroregeneration has a beneficial effect.

2. Use according to claim 1, wherein said disease is a neurodegenerative disorder, a neuroinflammatory disorder of the CNS, an acute ischemic brain lesion or hypoxic brain lesion.

3. Use according to claim 2, wherein said neurodegenerative disorder is Alzheimer's Disease, Parkinson's Disease, Creutzfeldt Jakobs Disease (CJD), Hallervorden Spatz Disease or Huntington's Disease.

4. Use according to claim 2, wherein said neuroinflammatory disorder is Multiple Sclerosis (MS).

5. Use according to any one of claims 1 to 4, wherein said compound is an antisense oligonucleotide reducing or inhibiting the expression of the gene encoding TGF-β1 and/or TGF-R.

6. Use according to any one of claims 1 to 4, wherein said compound is a compound reducing or inhibiting the binding of TGF-β1 to its receptor.

7. Use according to claim 6, wherein said compound is a neutralizing antibody directed against the TGF-β receptor II ($TGF\text{-}R_{II}$).

8. Use according to claim 6, wherein said compound is a soluble TGF-β receptor.

9. Use according to claim 8, wherein said soluble TGF-β receptor is the TGF-β receptor II (TGF-R_{II}).

10. A method for identifying a compound interfering with (a) the biological activity of TGF-β1 or its expression, or (b) the TGF-β1/TGF-R signaling, comprising the steps of:

(a) incubating a candidate compound with a test system comprising TGF-β1 and neuronal precursor cells; and

(b) assaying the expression of active TGF receptors or the proliferation of the neuronal precursor cells;

wherein

(c) an abolition of (i) the suppression of expression of active TGF receptors or (ii) suppression of proliferation of the neuronal precursor cells compared to the test system in the absence of said test compound is indicative of the presence of a candidate compound having the desired properties.

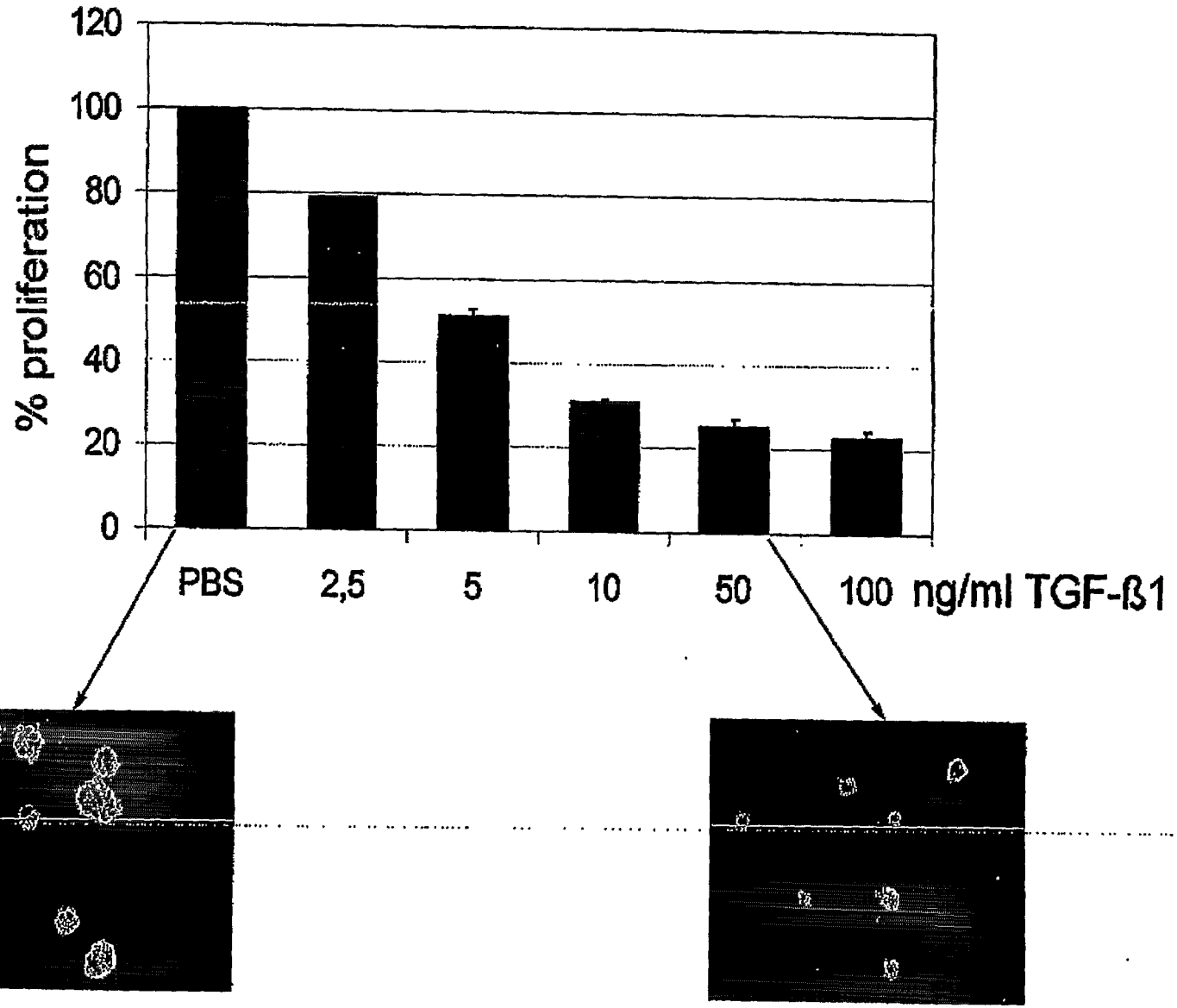
11. Use of a compound identified by the method of claim 10 for the preparation of a pharmaceutical composition for the prevention or treatment of a disease, wherein neurogenesis or neuroregeneration has a beneficial effect.

Abstract

Inhibitors of TGF-beta/TGF-R signaling for treatment of neurodegenerative diseases

Described is the use of a compound interfering with (a) the biological activity of TGF-beta1 or its expression, or (b) the TGF-beta1/TGF-R signaling, for the preparation of a pharmaceutical composition for the prevention or treatment of a disease, wherein neurogenesis and/or neuroregeneration has a beneficial effect, in particular a disease like Morbus Alzheimer, Morbus Parkinson, Creutzfeldt Jakobs Disease (CJD), Hallervorden Spatz Disease, Huntington's Disease, Multiple Sclerosis etc.

Figure 1



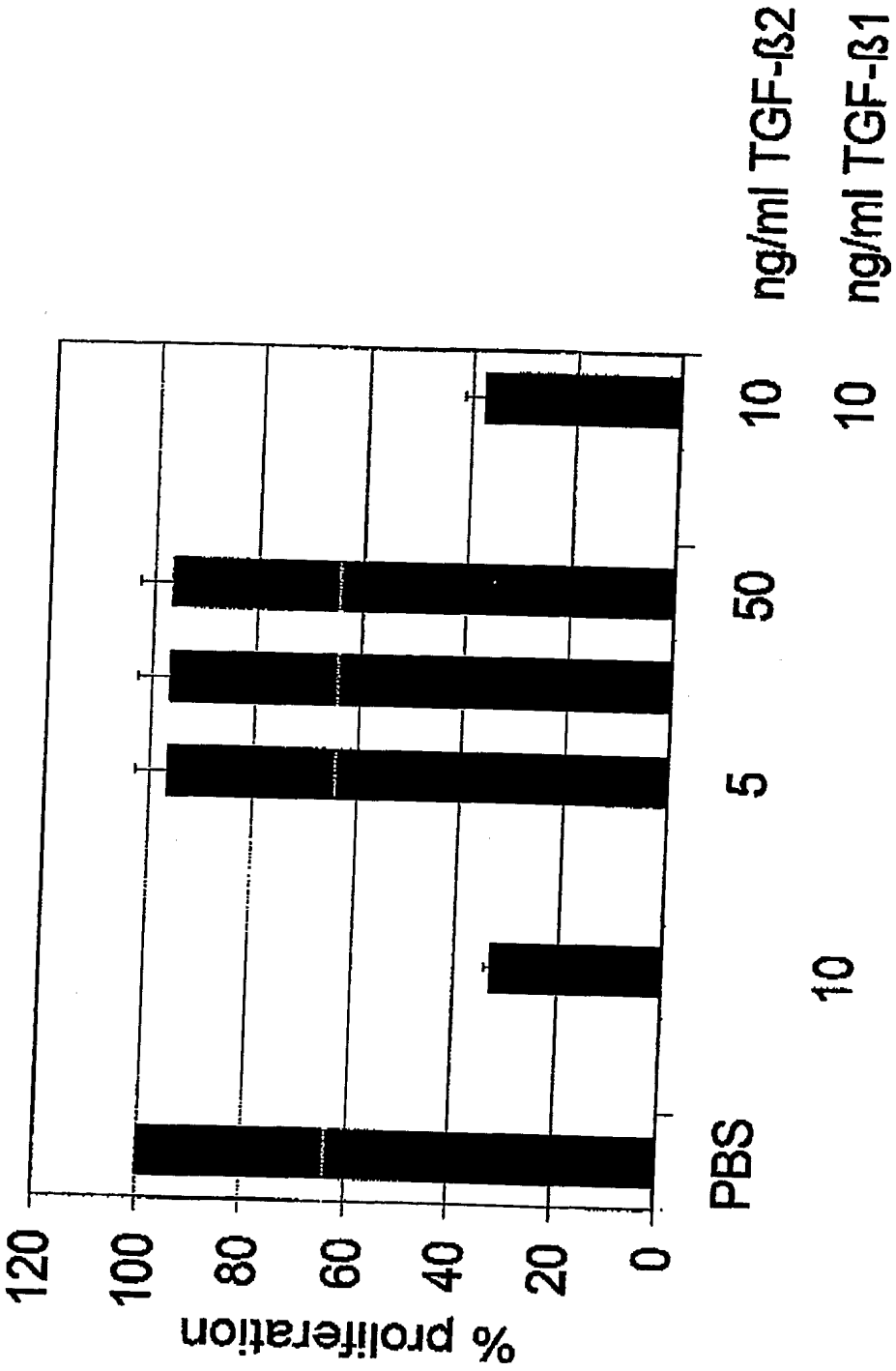

% proliferation
120
100
80
60
40
20
0
PBS
10
5
50
10
10
ng/ml TGF-ß2
ng/ml TGF-ß1

Figure 2

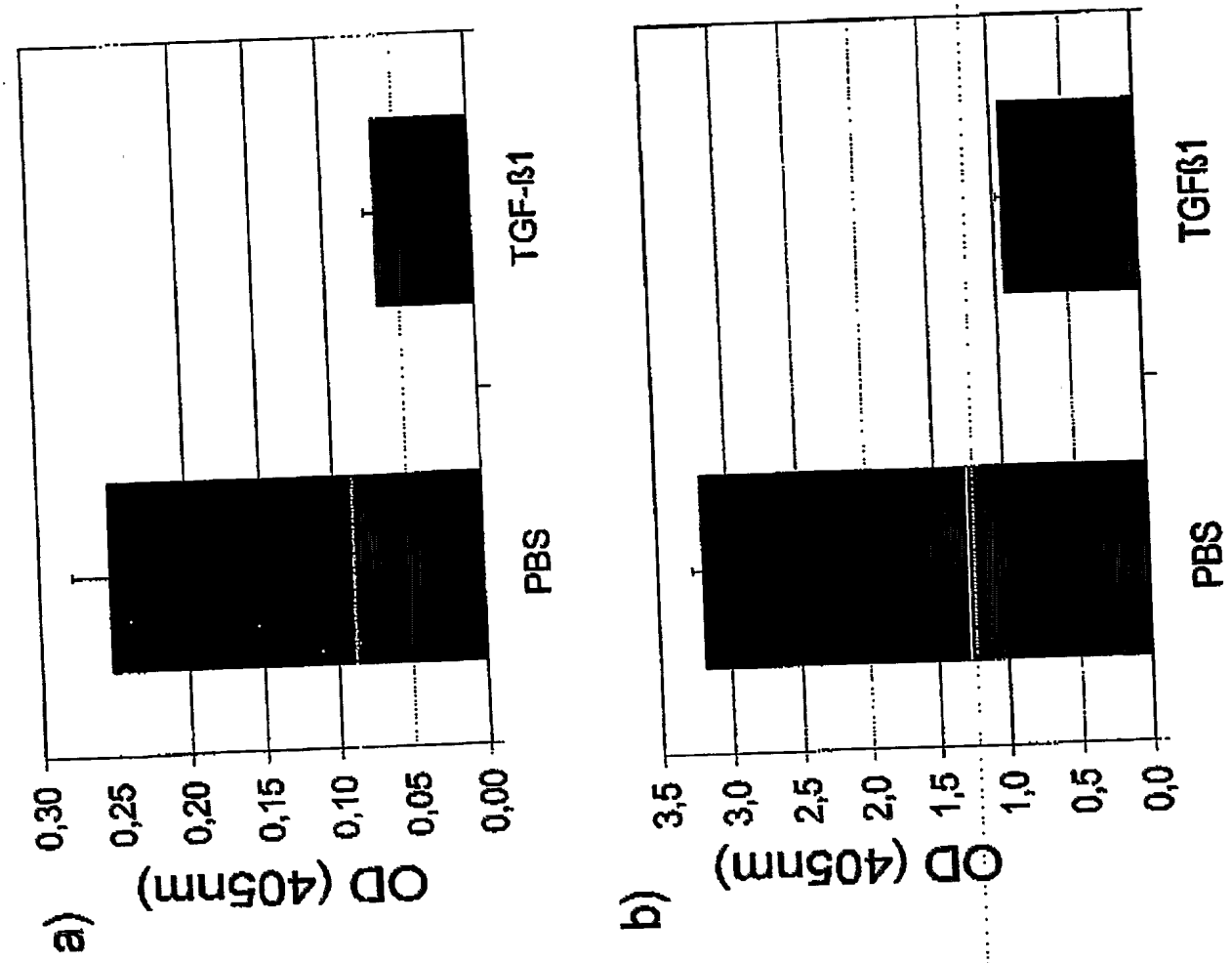

a)
OD (405nm)
0,30
0,25
0,20
0,15
0,10
0,05
0,00
PBS
TGF-ß1
b)
OD (405nm)
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
PBS
TGFß1

Figure 3

Figure 4

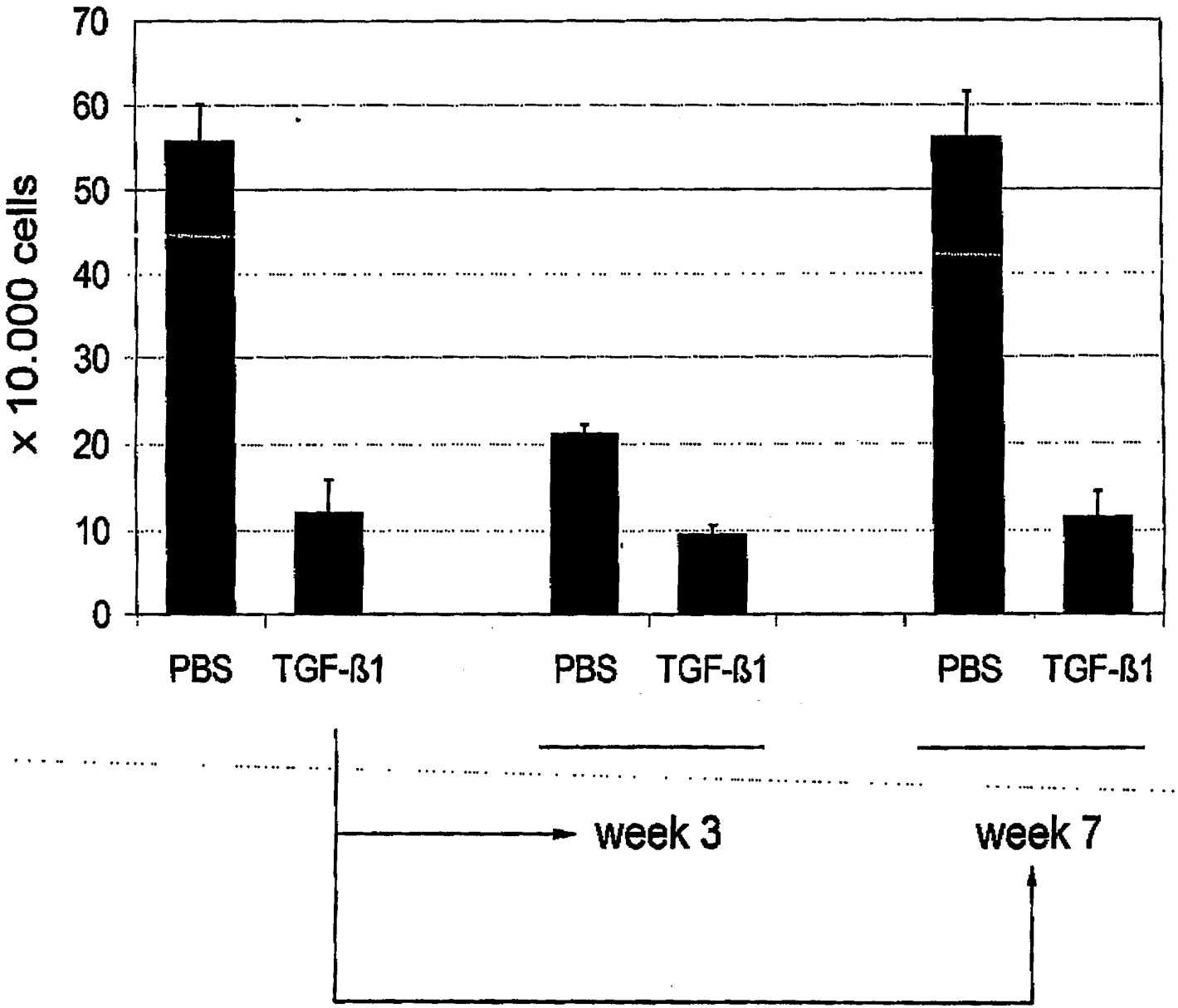


Figure 5

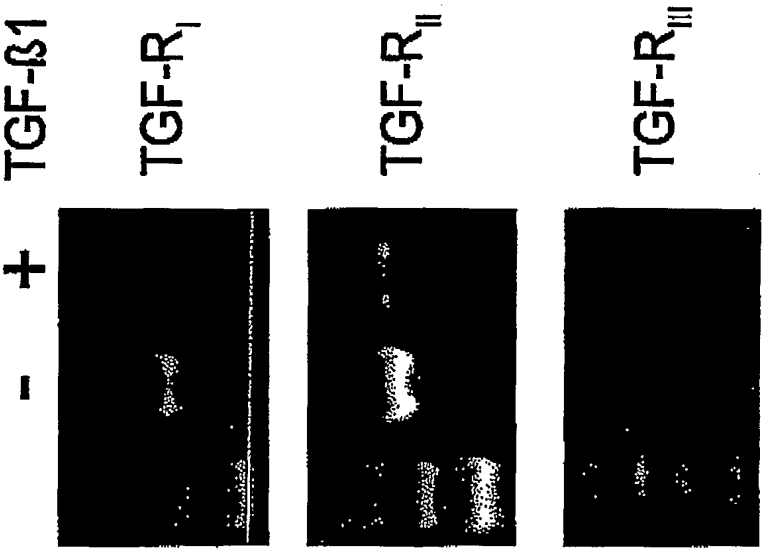


Figure 6

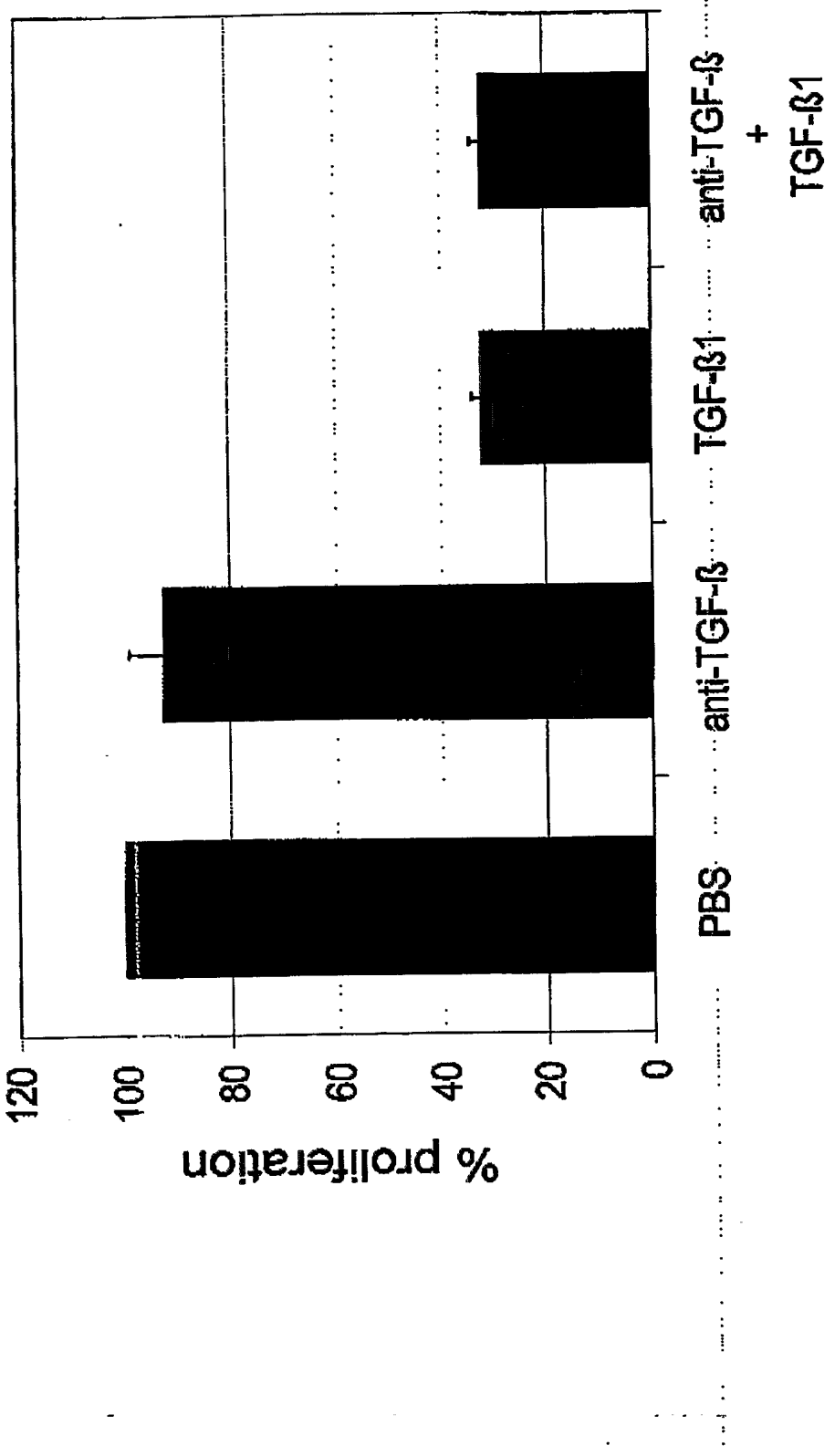

% proliferation
120
100
80
60
40
20
0
PBS
anti-TGF-ß
TGF-ß1
anti-TGF-ß
+
TGF-ß1

Figure 7

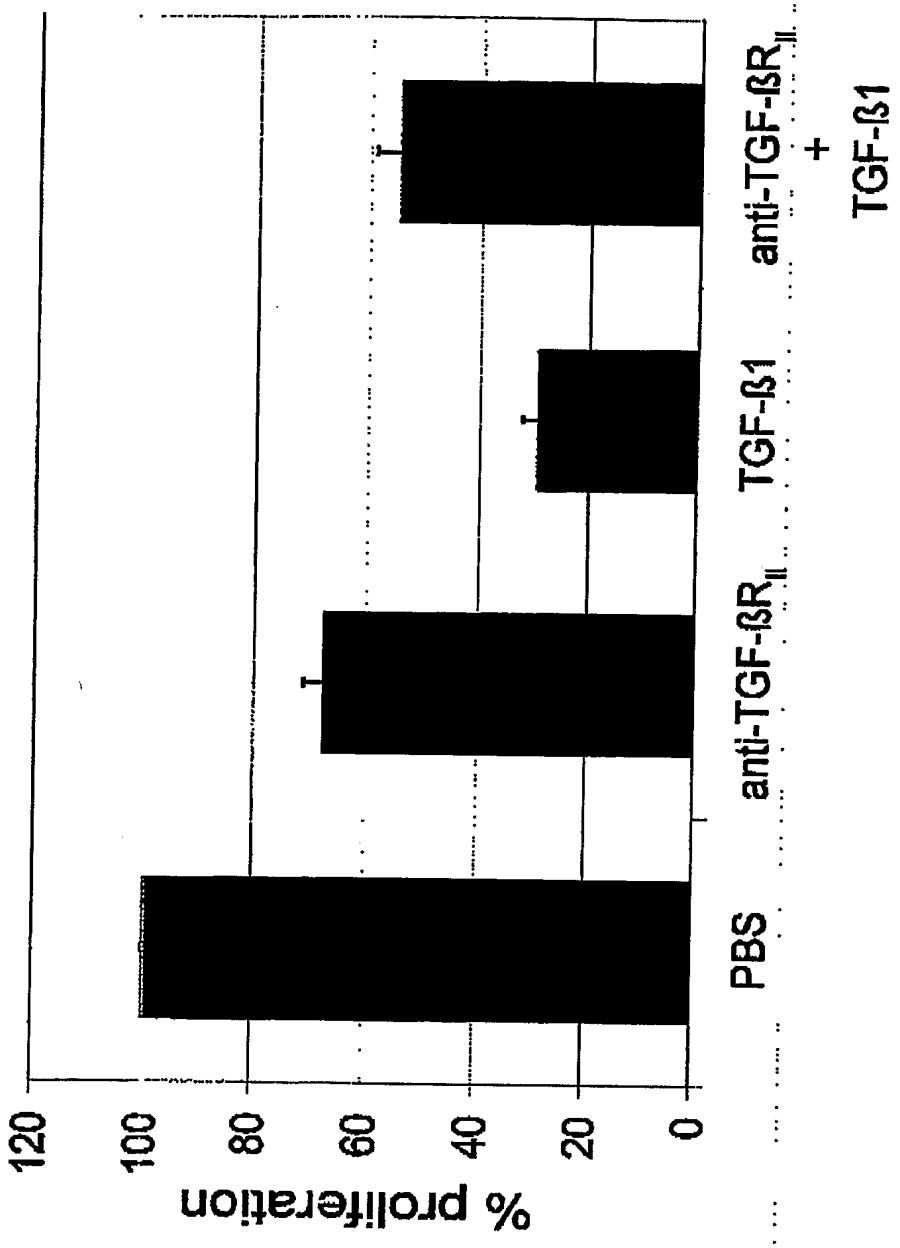

% proliferation
120
100
80
60
40
20
0
PBS
anti-TGF-βR$_{II}$
TGF-β1
anti-TGF-βR$_{II}$ + TGF-β1

Figure 8

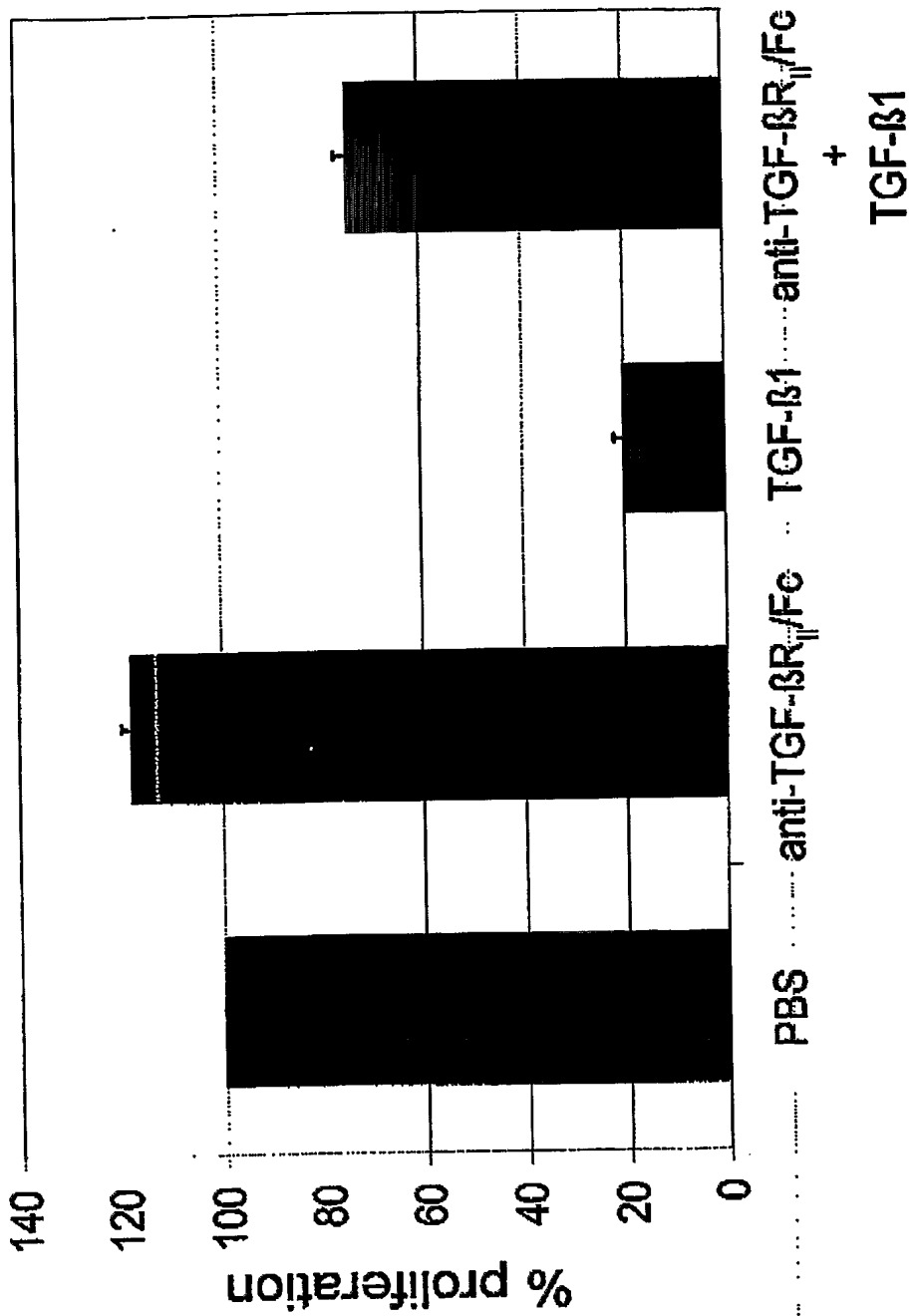
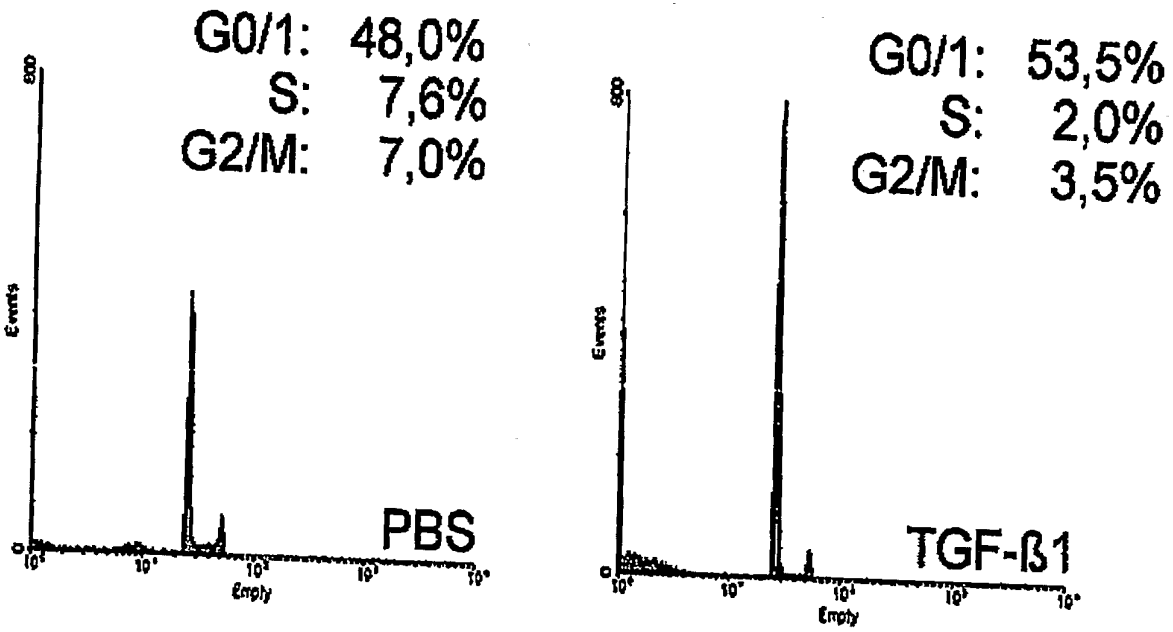

% proliferation
140
120
100
80
60
40
20
0
PBS
anti-TGF-βRII/Fc
TGF-β1
anti-TGF-βRII/Fc + TGF-β1

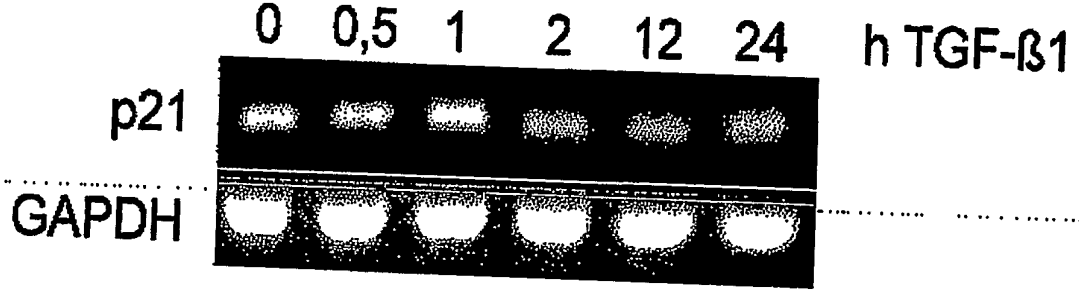
Figure 9

Figure 10

a)



b)



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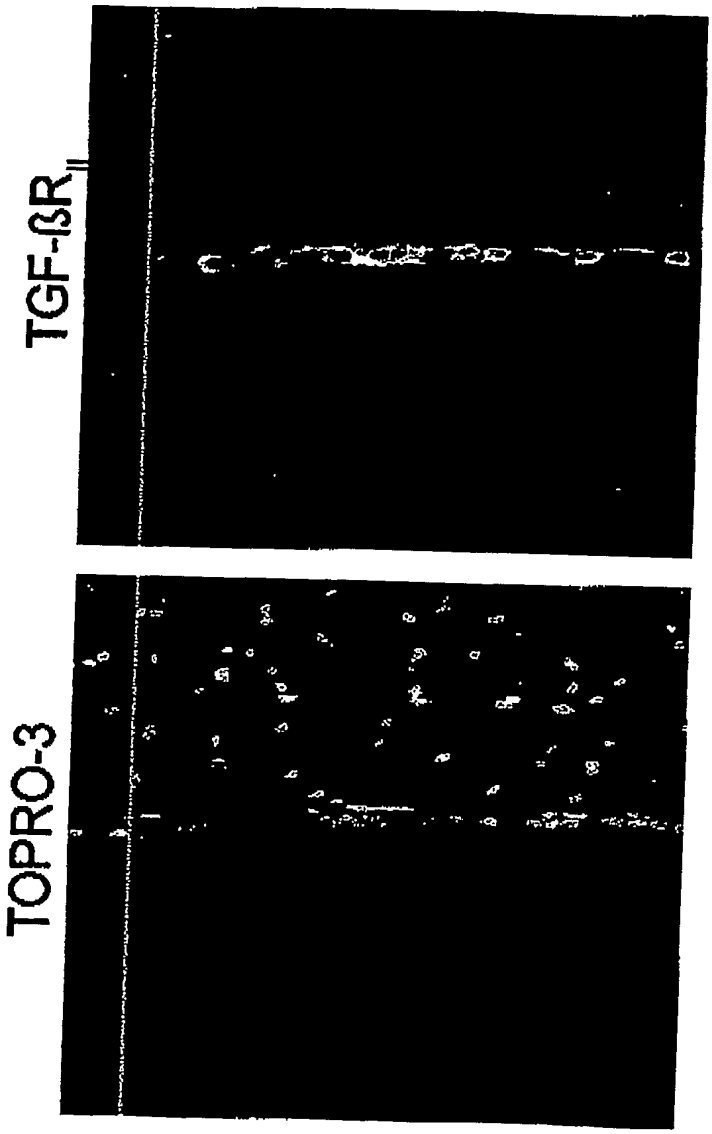
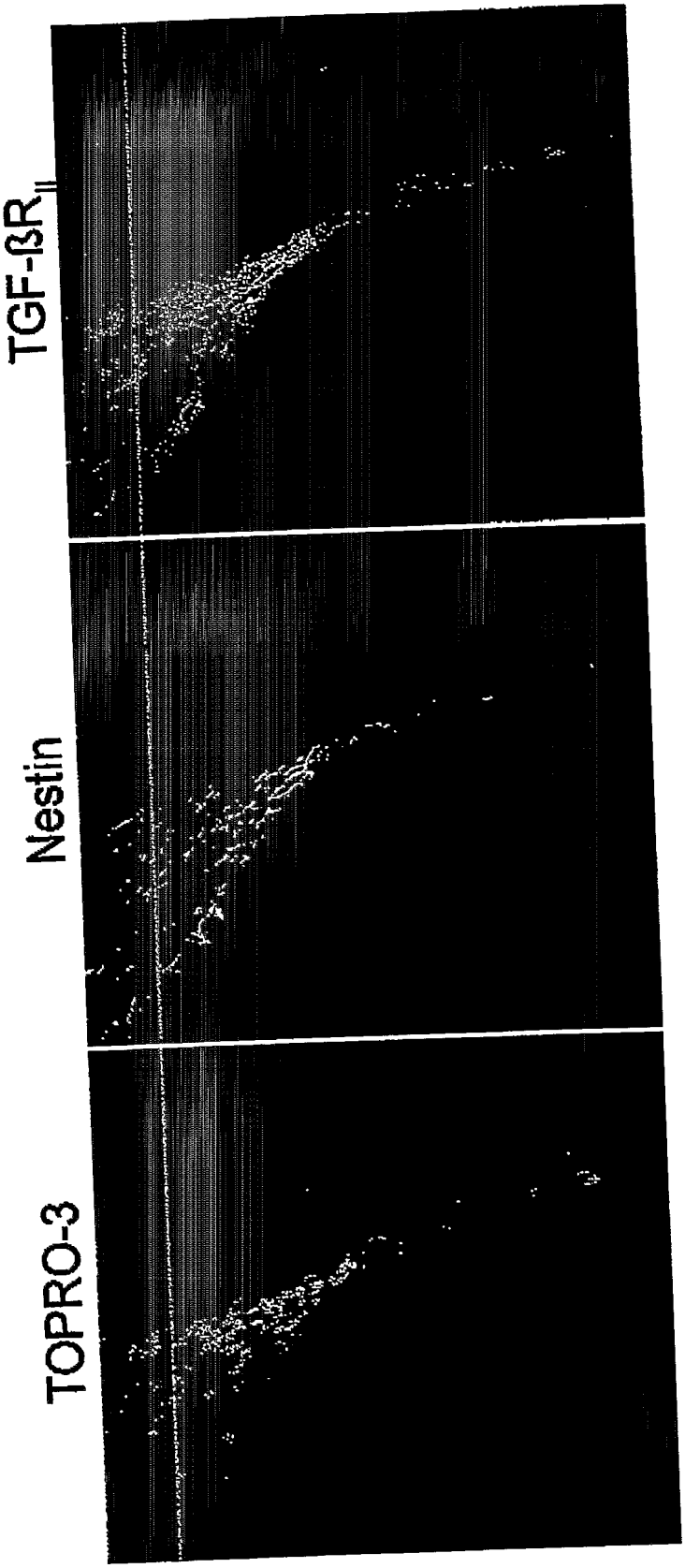


Figure 12



Figur 13

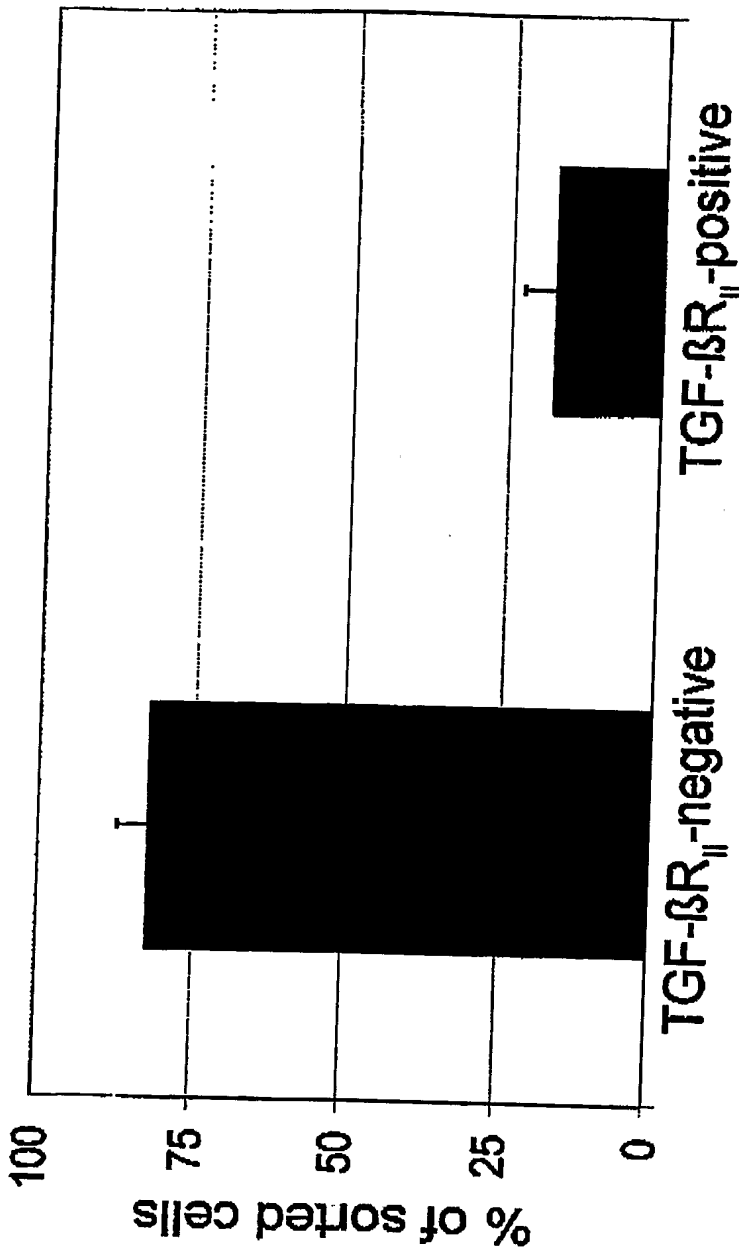

% of sorted cells
100
75
50
25
0
TGF-βR$_{II}$-negative
TGF-βR$_{II}$-positive

Figure 14

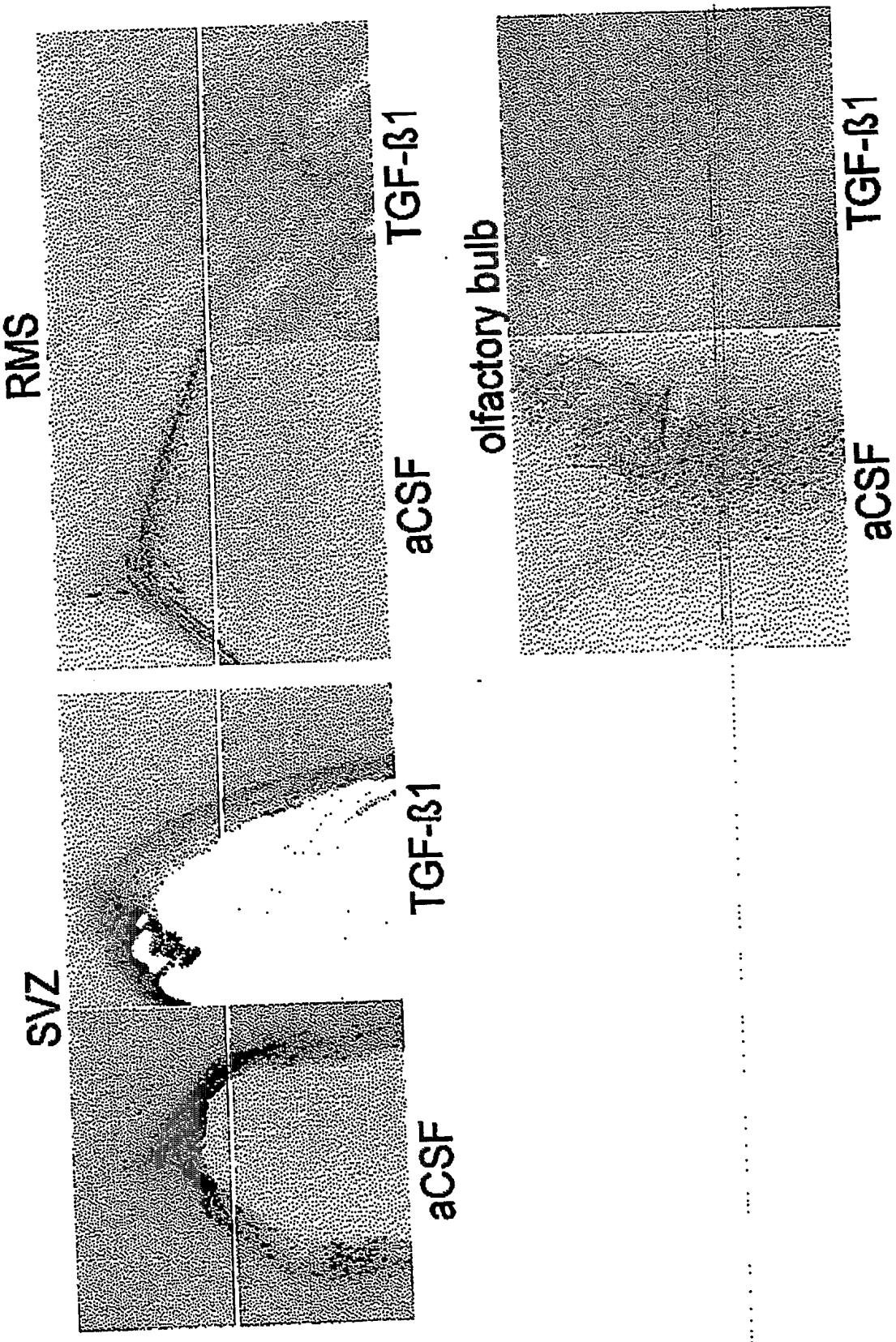


Figure 15